Research Article

Journal of PeptideScience

Received: 27 January 2010 Revised: 19 April 2010 Accepted: 20 April 2010 Published online in Wiley Interscience: 27 May 2010

(www.interscience.com) DOI 10.1002/psc.1252

# Synthesis of pathological and nonpathological human exon 1 huntingtin

**David Singer, Thomas Zauner, Maika Genz, Ralf Hoffmann and Thole Zuchner***

**Huntington's disease (HD) is a neurodegenerative disorder that affects approximately 1 in 10 000 individuals. The underlying gene mutation was identified as a CAG-triplet repeat expansion in the gene *huntingtin*. The CAG sequence codes for glutamine, and in HD, an expansion of the polyglutamine (poly-Q) stretch above 35 glutamine residues results in pathogenicity. It has been demonstrated in various animal models that only the expression of exon 1 huntingtin, a 67-amino acid-long polypeptide plus a variable poly-Q stretch, is sufficient to cause full HD-like pathology. Therefore, a deeper understanding of exon 1 huntingtin, its structure, aggregation mechanism and interaction with other proteins is crucial for a better understanding of the disease. Here, we describe the synthesis of a 109-amino acid-long exon 1 huntingtin peptide including a poly-Q stretch of 42 glutamines. This microwave-assisted solid phase peptide synthesis resulted in milligram amounts of peptide with high purity. We also synthesized a nonpathogenic version of exon 1 huntingtin (90-amino acid long including a poly-Q stretch of 23 glutamine residues) using the same strategy. In circular dichroism spectroscopy, both polypeptides showed weak alpha-helical properties with the longer peptide showing a higher helical degree. These model peptides have great potential for further biomedical analyses, e.g. for large-scale pre-screenings for aggregation inhibitors, further structural analyses as well as protein–protein interaction studies. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.**

*Supporting information may be found in the online version of this article*

**Keywords:** Huntington's disease; huntingtin; microwave-assisted peptide; synthesis, CD spectroscopy

## Introduction

Huntington's disease (HD) is an inheritable neurodegenerative disorder that affects approximately 1 in 10 000 individuals. Symptoms and signs include depression, personality changes, weight loss, dementia and severe motor disturbances before leading to death [1]. The underlying gene mutation was identified as a CAG-triplet repeat expansion in the gene *huntingtin* [2]. The protein huntingtin is 3144-amino acid long and is encoded over 67 exons with the CAG-triplet repeat located within the first exon. The CAG sequence codes for glutamine, and in HD, an expansion of the polyglutamine (poly-Q) stretch above 35 glutamines results in pathogenicity. The length of the poly-Q stretch inversely correlates with the age of onset of symptoms [1]. A repeat length of 40–55 glutamines typically leads to onset of symptoms at an age of 35–50 years, whereas any poly-Q stretch less than 35 glutamines does not lead to the disease [1].

Numerous animal and cell culture models for HD exist and it has been demonstrated that only the expression of exon 1 huntingtin, a 67-amino acid-long polypeptide plus a variable poly-Q stretch, is sufficient to cause full HD-like pathology in various animal models [3–6]. At the same time, expression of exon 1 huntingtin is sufficient to cause the typical formation of huntingtin aggregates, also found in brains of HD patients. Therefore, a deeper understanding of exon 1 huntingtin is crucial for a better understanding of the disease.

Several attempts have been undertaken to overexpress exon 1 huntingtin or longer parts of the protein for subsequent isolation and purification of the protein but have turned out to be extremely difficult, mainly due to cell toxicity of the pathological form of the protein and its high aggregation potential (Zuchner T, unpublished results). However, huntingtin with a nonpathological poly-Q stretch has been successfully overexpressed [6].

Isolation of huntingtin from cell or animal models is possible but difficult and the amount of obtainable protein is very low. In addition, huntingtin preparations from aggregates are extremely difficult to purify from other proteins contained in the aggregates and are not characterized regarding their phosphorylation status [7,8].

Parts of exon 1 huntingtin have been synthesized earlier, mostly focusing on the poly-Q stretch with some flanking sequences to study aggregation processes [9–12]. However, to the best of our knowledge, the pathogenic form of exon 1 huntingtin with more than 35 glutamines has never been synthesized before. Only one report studied the nonpathogenic form of exon 1 huntingtin with a poly-Q stretch of 35 glutamines without providing any details about its synthesis or further characterization [9]. This means that all successful attempts to synthesize exon 1 huntingtin have been restricted to the nonpathogenic form, which does not form aggregates.

* *Correspondence to: Thole Zuchner, Institute of Bioanalytical Chemistry, Center for Biotechnology and Biomedicine, Leipzig University, Deutscher Platz 5, 04103 Leipzig, Germany. E-mail: zuechner@rz.uni-leipzig.de*

*Institute of Bioanalytical Chemistry, Center for Biotechnology and Biomedicine, Leipzig University, Leipzig, Germany*

Journal of PeptideScience

Here, we describe the synthesis of a 109-amino acid-long exon 1 huntingtin peptide including a poly-Q stretch of 42 glutamines and a nonpathogenic version of exon 1 huntingtin (90-amino acid long including a poly-Q stretch of 23 glutamines). Both syntheses were achieved by microwave-assisted SPPS and resulted in milligram amounts of peptide with extremely high purity.

## Materials and Methods

### Peptide Synthesis

Synthesis of exon 1 huntingtin peptides, both the pathogenic (htt42Q) and the nonpathogenic (htt23Q) peptide, was carried out on the microwave-assisted peptide synthesizer Liberty (CEM, Matthews, NC, USA) using TentaGel R Trt-Proline (0.18 mmol/g) (RAPP Polymere GmbH, Tübingen, Germany). All side chains of trifunctional Fmoc amino acids were protected with 2,2,5,7,8-pentamethylchroman-6-sufonyl (Pbf) for Arg, $^{t}$Bu for Glu, Ser and Thr, trityl (Trt) for Gln and His, and *tert*-butyloxycarbonyl (Boc) for Lys (MultiSynTech GmbH, Witten, Germany and ORPEGEN Pharma GmbH, Heidelberg, Germany). All Fmoc amino acids were activated with HBTU/DIPEA and coupled in a fourfold molar excess with regard to resin substitution. The peptides were synthesized in a 100-µmol scale using recommended standard coupling protocols for microwave-assisted SPPS for the first 12 amino acids. All following amino acids were coupled twice for 5 min. For glutamine, the coupling time was elongated to 15 min per cycle with 5-min incubation at room temperature followed by 10-min incubation at 75 °C with a microwave power of 25 W. After synthesis, the resins were washed with DMF and methylene chloride, dried and cleaved with 2.5% water and 2.5% triisopropylsilane in TFA at room temperature for 3 h. The peptides were precipitated with cold diethyl ether, washed three times with diethylether and dried.

### Peptide Purification

Both peptides were purified on an Äkta HPLC System (Amersham Bioscience GmbH, Freiburg, Germany) using a Jupiter C18-column (21.2 × 250 mm, 10-µm particles, 300 Å pore size; Phenomenex Inc., Torrance, CA, USA) at a flow rate of 10 ml/min. After injection of two times 5 ml peptide solution, elution was performed by a linear gradient from 3 to 60% aqueous acetonitrile (ACN) in the presence of 0.1% TFA within 30 min.

For optimal purification, htt23Q and htt42Q were cleaved from the resin without final deprotection. Dry Fmoc-htt23Q was dissolved in 60% ACN (0.1% TFA) and diluted with 0.1% TFA to a final concentration of 12% ACN. The peptide was bound to the column and eluted with a linear gradient from 3 to 60% ACN in 30 min. Fractions containing Fmoc-htt23Q were pooled. For Fmoc cleavage, the dried peptide was dissolved in DMF and piperidine was added to a final concentration of 30%. After 20 min, htt23Q was precipitated with the tenfold volume of ice-cold diethylether. The residual was again dissolved in 60% ACN, diluted and purified as described above.

Due to solubility problems of Fmoc-htt42Q, a modified purification protocol was used. After cleavage of the Fmoc group from the crude peptide Fmoc-htt42Q as described above, the peptide was dissolved in 1 ml TFA. After sonification, 60% aqueous ACN (1 ml) was added and the clear solution was diluted fourfold with water and purified by RP-HPLC as described above using a linear gradient with an increase of 1% eluent B per minute.

The purity of the peptides was confirmed by analytical RP-HPLC using a Jupiter C18-column (150 × 2 mm, Phenomenex Inc.) with a linear gradient form 3 to 60% aqueous ACN (0.1% TFA) with an increase of 2% eluent B per minute at a flow rate of 0.2 ml/min. Crude peptides were analyzed using the same column with a diameter of 4.6 mm. Separation was performed at 1 ml/min with an linear gradient from 3 to 60% aqueous ACN (0.1% TFA) in 30 min.

### Mass Spectrometry

Peptide masses were confirmed by electro spray ionization mass spectrometry (ESI-MS; PE SCIEX, API QSTAR Pulsar; ESI-QqTOF-MS; Applied Biosystems GmbH, Darmstadt, Germany) equipped with a nano-ESI source (Proxeon, Odense, Denmark). Peptides were dissolved in 60% aqueous ACN containing 1% formic acid and transferred to nano-spray capillaries (Proxeon). Emitter voltage was 1100 V and the gas pressure 6 psi. Data analysis was done with Analyst QS 1.0 and Bioanalyst QS 1.0 (Applied Biosystems).

### Circular Dichroism Spectroscopy

Circular dichroism (CD) spectra were recorded on a JASCO J720A spectropolarimeter using a quarz cell cuvette with 2.0-mm path length at 20 °C under nitrogen atmosphere. Peptide solutions (25 µM) were prepared in phosphate buffer (10 mM pH 7.4). The secondary structure was stabilized with 2,2,2-trifluorethanol at a final concentration of 50% (v/v). Spectra were recorded from 190 to 250 nm with a bandwidth of 1.0 nm and a rate of 0.5 nm/s. Five scans were averaged and corrected for the background of the buffer solution. Ellipticity is reported as mean residue ellipticity [$\theta$].

## Results and Discussion

The synthesis of human exon 1 huntingtin is challenging. The peptides are around 100-amino acid long and contain two stretches, which are difficult to obtain in peptide synthesis: a polyglutamine stretch as well as a proline-rich region located *C*-terminally of the polyglutamine stretch. *In silico* predictions of the peptides regarding their accessibility toward peptide synthesis confirm these expected problems (Peptide Companion, CSPS Pharmaceuticals Inc., San Diego, CA, USA).

Initial trials to synthesize exon 1 huntingtin relied on a chemical ligation strategy [13]. In one of the attempts, we synthesized two different fragments of huntingtin (*N*-terminal huntingtin with seven glutamines and a 30 amino acid residues-long *C*-terminal fragment) by Fmoc/$^{t}$Bu chemistry on a SYRO 2000 peptide synthesis robot. All attempts regarding the subsequent ligation of both fragments failed due to difficulties that were mainly attributed to the BOC protection of lysine side chains and subsequently resulted in very low yields.

Due to the problems using chemical ligation strategies and promising results published before in this area for long peptides [14], a microwave-assisted peptide synthesis robot was acquired. The subsequent microwave-assisted peptide synthesis was performed on a Liberty system (CEM) using the conditions described in Section on Materials and Methods for the peptide sequences given in Table 1.

For the synthesis of both peptides, the TentaGel R Trt-Proline resin was used for two reasons. First was to provide space for the long peptide chain by the low loading of 0.18 mmol/g and the



  

**Table 1.** Sequences of the synthesized peptides

| Peptide | Sequence |
|---|---|
| httQ23 | MATLEKLMKAFESLKSF$\mathbf{Q_{23}}P_{11}$QLPQPPPQAQPLLPQPQP$_{10}$ GPAVAEEPLHRP |
| httQ42 | MATLEKLMKAFESLKSF$\mathbf{Q_{42}}P_{11}$QLPQPPPQAQPLLPQPQP$_{10}$ GPAVAEEPLHRP |

httQ23: exon 1 huntingtin with a 23-glutamine-long poly-Q stretch; htt42Q: exon 1 huntingtin with a 42-glutamine-long poly-Q stretch.

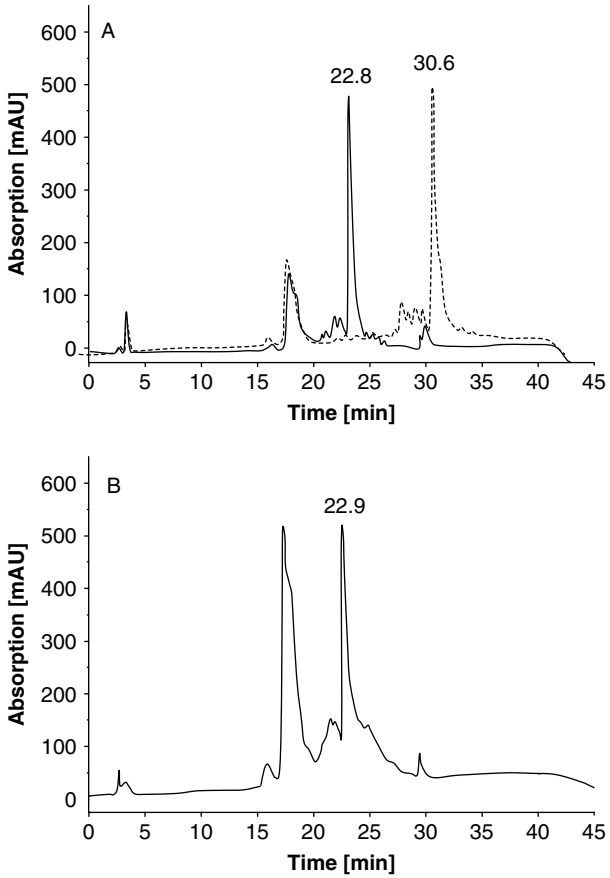


**Figure 1.** RP chromatograms of the crude peptides Fmoc-htt23Q (A, dashed line), htt23Q (A, solid line) and htt42Q (B). All retention time labeled peaks represent the correlative peptide. Separation was done using a Jupiter $C_{18}$-column (150 × 4.6 mm, 5 µm, 300 Å) at a flow rate of 1 ml/min with a linear gradient from 3 to 60% ACN in the presence of 0.1% TFA in 30 min. Absorption was recorded at 220 nm.

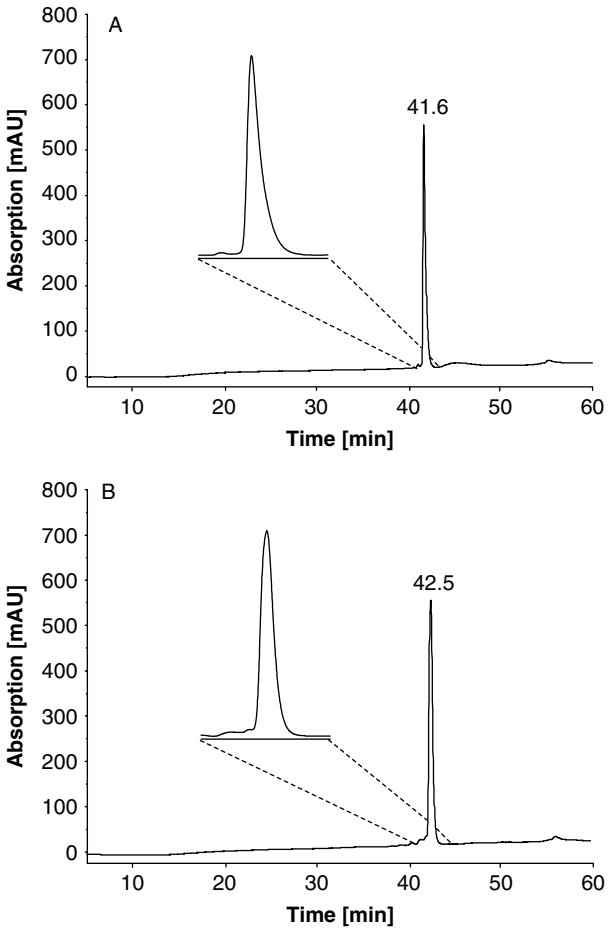


**Figure 2.** RP chromatograms of the purified synthetic human exon 1 huntingtin peptides containing (A) 23 (htt23Q) and (B) 42 glutamine residues (htt42Q). A Jupiter $C_{18}$-column (150 × 2 mm, 3 µm, 300 Å) was used at a flow rate of 0.2 ml/min. After injection of 5 µg of each peptide, elution was performed by a linear gradient from 3 to 60% ACN in the presence of 0.1% TFA with an increase of 2% per minute. Absorption was recorded at 214 nm.

excellent swelling properties in organic solvents. The second reason was an extensive diketopiperazine formation when a Proline-Wang resin was used. This side reaction was even enhanced by the high temperatures in the microwave synthesizer. Due to the predicted difficulties for the synthesis of the poly-Q stretches, all glutamines were coupled twice with an elongated coupling of 15 min with 10 min at 75 °C. The first 5 min at room temperature were used to ensure a good soaking of the resin with the amino acid and all coupling reagents before the reaction mixture was heated. This strategy is comparable with the standard arginine coupling cycles where even a 30-min incubation at room temperature is used for optimal diffusion of the reagents.

The first peptide, exon 1 huntingtin with a 23-glutamine-long poly-Q stretch (htt23Q), was first purified as Fmoc derivative to exploit the hydrophobic shift induced by the Fmoc group. Thereby undesired peptide fragments coeluting with the Fmoc-deprotected htt23Q could be separated increasing the purity of the final product. After Fmoc cleavage, the retention time shifted from 30.6 to 22.6 min (Figure 1(A)) and htt23Q was purified a second time. This two-step purification procedure was not applicable to the second peptide, exon 1 huntingtin with a 42-glutamine-long poly-Q stretch (htt42Q), due to the dramatically reduced solubility of Fmoc-htt42Q. Therefore, the Fmoc group was cleaved but the peptide was still not soluble even in 60% ACN. Solubilization was then accomplished by dissolving the peptide in pure TFA and dilution with ACN/water (Figure 1(B)). Htt42Q was purified using a linear gradient with an increase of 1% B per minute. Both purified peptides htt23Q and htt42Q eluted under analytical conditions using a linear gradient of 2% eluent B per minute as single, sharp peaks after 41.6 and 42.9 min, respectively (Figure 2(A) and (B)). Despite the limited resolution of RP-HPLC for such long synthetic peptides, the peak shapes indicated the high purity of both synthesized peptides.



  

Journal of PeptideScience

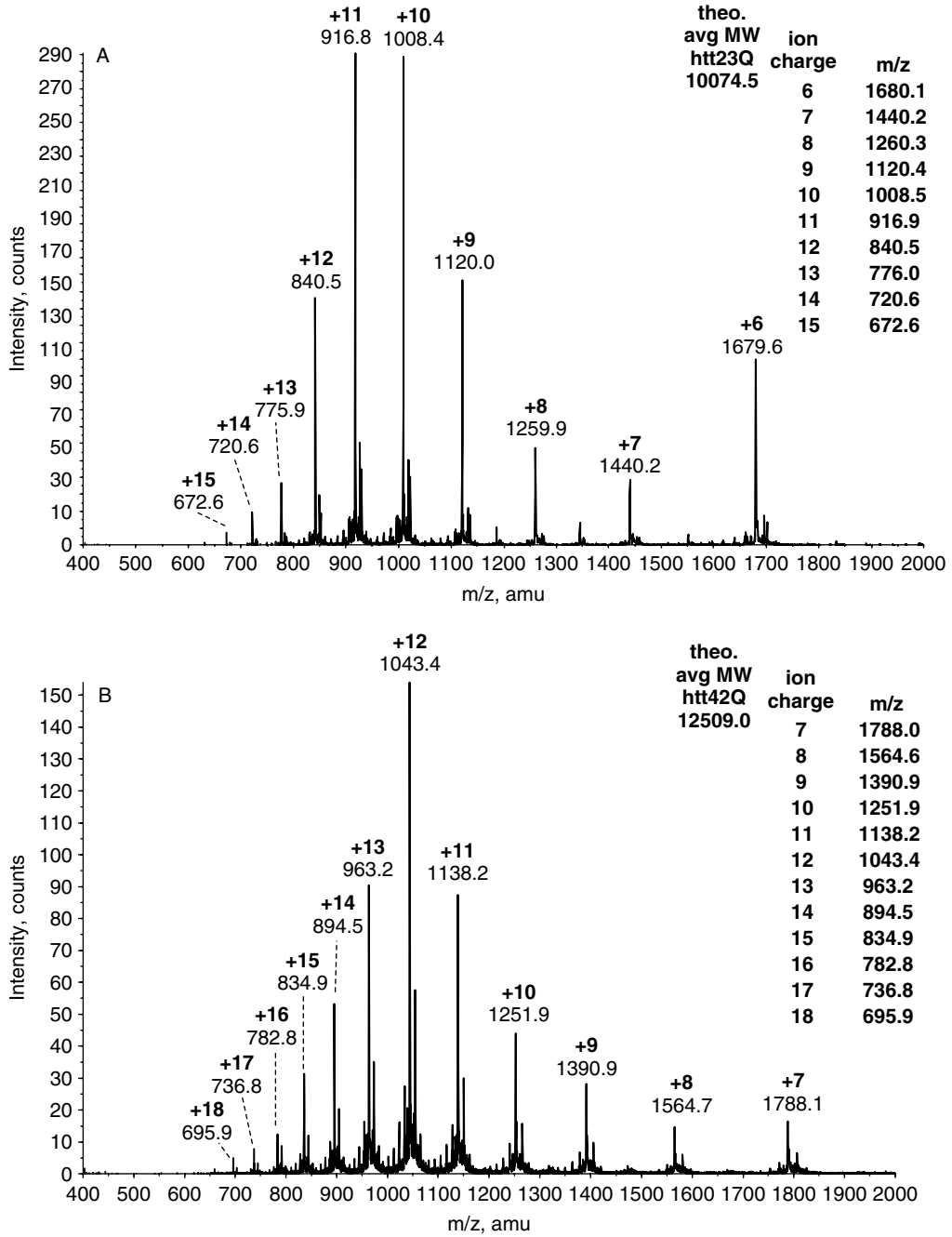


**Figure 3.** ESI-MS spectra of the purified peptides htt23Q (A) and htt42Q (B), respectively. The charge series with the indicated charge states and *m/z* values confirmed the theoretical average masses of 10 074.5 Da for htt23Q and 12509.0 Da for htt42Q without observing significant by-products.

The purified peptides were further analyzed by electrospray ionization mass spectrometry using an ESI-QqTOF-MS. The obtained charge series confirmed the theoretical average masses of both products, in particular 10 074.5 for htt23Q (Figure 3(A)) and 12 509.0 for htt42Q (Figure 3(B)). Furthermore, only the mass spectrum for Htt42Q shows a minor side product which has been identified as htt43Q. As the peak intensity of this side product is rather weak and the identity of the side product itself is not critical for the further use of the peptide in this study, we did not perform additional purification steps.

CD spectroscopy was performed to study the secondary structures of both polypeptides. CD spectra were recorded in either phosphate buffer or the same buffer in the presence of 50% TFE to stabilize secondary structures.

The exact peptide amounts used for CD were determined by integrating the peak area in HPLC. In phosphate buffer, both huntingtin versions showed identical CD spectra (Figure 4(A)), which also did not change significantly over time. The minima of both curves are located at 203 nm indicating a stabilized turn [type I (III)].

In the presence of 50% TFE (Figure 4(B)), both polypeptides showed weak alpha-helical properties with htt42Q forming a more pronounced alpha-helical structure. The band at 192 nm for htt42Q showed a hypsochromic shift (1 nm) in the htt23Q and was



  

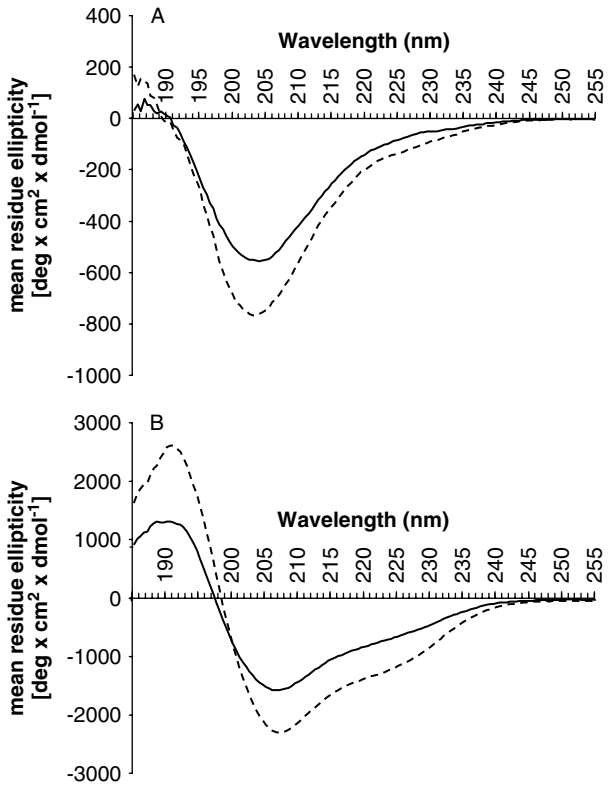


**Figure 4.** CD spectra of htt23Q (solid line) and htt42Q (dashed line) recorded in (A) phosphate buffer (10 mmol/l, pH 7.4) and (B) in the same buffer in the presence of 50% TFE. The peptide quantities were determined by their peak areas and the acquired spectra were corrected accordingly.

also more intense for htt42Q. The same applies for the maximum at 208 nm (or 206 nm, respectively). As the intensity ratios of the maxima at 222 and 208 nm (and 206 nm, respectively) of both spectra did not show the expected ratio of approximately 1 : 1, both alpha-helices were not fully developed. Beta-sheet structures can be excluded for both versions of huntingtin in both the absence and presence of TFE, as neither a maximum at 198 nm nor a minimum at 217 nm was observed.

The data of htt23Q are in good agreement with a recent report on nonpathogenic huntingtin fragments that adapted alpha-helical confirmations [15]. At the same time, the relatively similar confirmation of htt42Q with the absence of beta-sheet structures was surprising when looking at *in silico* predictions for pathogenic huntingtin fragments or data published for other polyglutamine-containing proteins and peptides [16]. However, Darnell *et al*. [12] stated, based on their results obtained with shorter peptides, that 'flanking polyproline sequences inhibit beta-sheet structure in polyglutamine segments'. As htt42Q includes such a polyproline sequence *C*-terminal of the poly-Q stretch, the absence of a beta-sheet is in good agreement with these results.

Both peptides have great potential for further biomedical analyses. For instance, small fluorophores like rhodamine or fluorescein could be included into the peptide to study protein–protein interaction using fluorescence resonance energy transfer (FRET). In addition, phosphorylated versions of exon 1 huntingtin could be designed specifically to further study the role of phosphorylation, e.g. on its degradation mechanism [17]. Last but not least, both versions of exon 1 huntingtin could be used for the further investigation of the neuroprotective effect against excitotoxicity of exon 1 huntingtin [18,19].

## Conclusions

The direct synthesis of pathogenic as well as nonpathogenic exon 1 huntingtin with lengths of 109 and 90 amino acid residues was surprisingly simple and straightforward with the microwave-assisted synthesis protocol. Both huntingtin peptides were synthesized in high purities and quantities as confirmed by RP-HPLC and ESI-MS. CD spectroscopy of both peptides did not reveal conformational differences, although htt42Q showed a more pronounced alpha-helical structure than htt23Q.

The huntingtin polypeptides synthesized here have great potential for further biomedical analysis. They could be used, e.g., for large-scale pre-screenings for aggregation inhibitors or further structural analyses. Most importantly, both peptides could be modified as desired by the user.

### Acknowledgements

We would like to thank Anne Hansen for help with peptide synthesis and Dr Andrew Hagan for proofreading. We gratefully acknowledge the expert technical assistance of Katrin Müller. This work was supported by BMBF Innoprofile, Project Number 03IP604. We would also like to thank the group Prof. Annette G. Beck-Sickinger, Institute of Biochemistry, Leipzig University for the opportunity to perform CD spectroscopy on their instrument.

### Supporting information

Supporting information may be found in the online version of this article.

## References

1 Vonsattel JP, DiFiglia M. Huntington disease. *J. Neuropathol. Exp. Neurol.* 1998; **57**: 369–384.
2 The Huntington's Disease Collaborative Research Group. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* 1993; **72**: 971–983.
3 Mangiarini L, Sathasivam K, Seller M, Cozens B, Harper A, Hetherington C, Lawton M, Trottier Y, Lehrach H, Davies SW, Bates GP. Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell* 1996; **87**: 493–506.
4 Davies SW, Turmaine M, Cozens BA, DiFiglia M, Sharp AH, Ross CA, Scherzinger E, Wanker EE, Mangiarini L, Bates GP. Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. *Cell* 1997; **90**: 537–548.
5 Bates GP, Mangiarini L, Davies SW. Transgenic mice in the study of polyglutamine repeat expansion diseases. *Brain Pathol.* 1998; **8**: 699–714.
6 Li SH, Cheng AL, Li H, Li XJ. Cellular defects and altered gene expression in PC12 cells stably expressing mutant huntingtin. *J. Neurosci.* 1999; **19**: 5159–5172.
7 Qin ZH, Wang Y, Sapp E, Cuiffo B, Wanker E, Hayden MR. Huntingtin bodies sequester vesicle-associated proteins by a polyproline-dependent interaction. *J. Neurosci.* 2004; **24**: 269–281.
8 Landles C, Bates GP. Huntingtin and the molecular pathogenesis of Huntington's disease. *EMBO Rep.* 2004; **5**: 958–963.
9 Thakur AK, Jayaraman M, Mishra R, Thakur M, Chellgren VM, Byeon IJ, Anjum DH, Kodali R, Creamer TP, Conway JF, Gronenborn AM, Wetzel R. Polyglutamine disruption of the huntingtin exon 1 N terminus triggers a complex aggregation mechanism. *Nat. Struct. Mol. Biol.* 2009; **16**: 380–389.

  

10 Bhattacharyya A, Thakur AK, Chellgren VM, Thiagarajan G, Williams AD, Chellgren BW. Oligoproline effects on polyglutamine conformation and aggregation. *J. Mol. Biol.* 2006; **355**: 524–535.
11 Ignatova Z, Thakur AK, Wetzel R, Gierasch LM. In-cell aggregation of a polyglutamine-containing chimera is a multistep process initiated by the flanking sequence. *J. Biol. Chem.* 2007; **282**: 36736–36743.
12 Darnell G, Orgel JPRO, Pahl R, Meredith SC. Flanking polyproline sequences inhibit $\beta$-sheet structure in polyglutamine segments by inducing PPII-like helix structure. *J. Mol. Biol.* 2007; **374**: 688–704.
13 Teruya K, Murphy AC, Burlin T, Appella E, Mazur SJ. Fmoc-based chemical synthesis and selective binding to supercoiled DNA of the p53 C-terminal segment and its phosphorylated and acetylated derivatives. *J. Pept. Sci.* 2004; **10**: 479–493.
14 Hill A, Boland M, Karas J, Wall V, Barnham K, Collins S, Scanlon D. Investigation of the structure of the N-terminal region of prion protein (PrP) via the microwave synthesis of peptide fragments up to 111 amino acids in length. *J. Pept. Sci.* 2008; **14**: (Suppl.): 90–91.
15 Kim MW, Chelliah Y, Kim SW, Otwinowski Z, Bezprozvanny I. Secondary structure of Huntingtin amino-terminal region. *Structure* 2009; **17**: 1205–1212.
16 Nagai Y, Popiel HA. Conformational changes and aggregation of expanded polyglutamine proteins as therapeutic targets of the polyglutamine diseases: exposed beta-sheet hypothesis. *Curr. Pharm. Des.* 2008; **14**: 3267–3279.
17 Thompson LM, Aiken CT, Kaltenbach LS, Agrawal N, Illes K, Khoshnan A, Martinez-Vincente M, Arrasate M, O'Rourke JG, Khashwji H, Lukacsovich T, Zhu YZ, Lau AL, Massey A, Hayden MR, Zeitlin SO, Finkbeiner S, Green KN, LaFerla FM, Bates G, Huang L, Patterson PH, Lo DC, Cuervo AM, Marsh JL, Steffan JS. IKK phosphorylates Huntingtin and targets it for degradation by the proteasome and lysosome. *J. Cell Biol.* 2009; **187**: 1083–1099.
18 Hansson O, Petersen A, Leist M, Nicotera P, Castilho RF, Brundin P. Transgenic mice expressing a Huntington's disease mutation are resistant to quinolinic acid-induced striatal excitotoxicity. *Proc. Nat. Acad Sci U. S. A.* 1999; **96**: 8727–8732.
19 Zuchner T, Brundin P. Mutant huntingtin can paradoxically protect neurons from death. *Cell Death Differ.* 2008; **15**: 435–442.



  