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Study of Solid-Phase Synthesis and Purification Strategies for the Preparation of Polyglutamine Peptides

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Received 30 December 2006; revised 11 April 2007; accepted 14 May 2007
Published online 21 May 2007 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bip.20763

ABSTRACT:

Many neurodegenerative diseases are related to an abnormal expansion of the CAG trinucleotide that produces polyglutamine segments in several proteins. However, the pathogenesis of these neurodegenerative states is not yet well understood. Thus, to evaluate the molecular mechanisms leading to those diseases, suitable research tools such as synthetic polyglutamine peptides are required. The synthesis and purification of such peptides are usually difficult because of poor solubility, which leads to low coupling and/or deblocking reactivity. After exploring many synthesis, solubilization and purification approaches, a protocol allowing the production of polyglutamines in good yield and high purity was developed. With this protocol, peptides of 10–30 glutamine residues were synthesized using a linear solid-phase strategy combined with a maximal side-chain protection scheme using fluorenylmethyloxycarbonyl (Fmoc) chemistry. After cleavage of the peptide from the polymeric support, the crude material was treated with glacial acetic acid and lyophilized. This treatment significantly improved the solubility of the polyglutamine peptides thus allowing their dissolution in aqueous conditions and purification through reverse-phase high performance liquid chromatography. These solubilization and purification conditions led to the formation of N-pyroglutamyl peptide derivatives that were easily isolated. These N-pyroglutamylated compounds also appear as useful research tools because data from the literature suggest that N-terminal modification of polyglutamine segments might play a role in their pathogenic properties.

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Keywords: peptide; polyglutamine; solid-phase synthesis; HPLC; purification; solubilization; aggregation; pyroglutamyl; neurodegeneration

This article was originally published online as an accepted preprint. The "Published Online" date corresponds to the preprint version. You can request a copy of the preprint by emailing the Biopolymers editorial office at biopolymers@wiley.com

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Contract grant sponsors: Canadian Institutes of Health Research; Natural Sciences and Engineering Research Council of Canada

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INTRODUCTION

A large number of neurodegenerative diseases, such as type 1, 2, 3, 6, 7, 12, and 17 spinocerebellar ataxias, type 2 episodic ataxia, Huntington disease, as well as dentatorubral-pallidoluysian and bulbomuscular atrophies share the same abnormal genetic CAG trinucleotide expansion.[1] Gene expression leads to an insertion of a glutamine homopolymer (polyQ) fragment in the corresponding protein. It remains to be clarified whether it is the mutant protein or some of its fragments

that are leading to the neurotoxic process that eventually will produce neurodegeneration. PolyQ motifs have a propensity to aggregate into supramolecular structures. It has been suggested that this process results from reduced solubility of a β-sheet assembly characterized by cooperative reinforcement of intermolecular hydrogen bonds involving amide side-chains.[2–4] However, a polyproline type II structure was also recently reported.[5] Furthermore, this intrinsic tendency to aggregate correlates with the poor solubility of polyQ peptides, which complicates any study involving these molecules. In fact, a rather small number of studies have been performed on polyQ peptides considering their pathophysiological importance. Strategies must be devised to circumvent the aggregation problems either at the synthesis or at the purification step. Unfortunately, these strategies were found either to interfere with the aggregation behavior of polyQ peptides or to be incompatible with a structural analysis.

Synthetic approaches used to produce polyQ peptides can be divided into two major categories: use of engineered microorganisms and chemical syntheses. The first class of synthetic approaches involves the use of polyQ-fusion proteins.[1,6,7] However, it has been shown that the choice of the specific carrier protein as well as the location of the inserted polyQ stretch within the protein may influence the properties of the glutamine repeats under investigation.[1] Proteolytic cleavage of glutathione S-transferase Huntington disease (GST-HD) fusion proteins was also developed to produce polyQ.[8,9] However, the cleavage of the carrier protein does not lead to the isolation of the polyQ stretch alone but contains a *C*-terminal oligoproline fragment that was shown to influence the polyQ aggregation and conformation.[5,10,11] The second class of synthetic approaches consists of producing polyQ by chemical reactions. Polymerization allows the production of high molecular weight homopolymers, but this approach leads to high polydispersity that is not compatible with most structural studies.[12–14] This problem can be partially overcome by using a solid-phase peptide synthesis approach on low substitution resins. However, the lack of solubility of growing polyQ chains, resulting in incomplete *N*-terminal deprotections and couplings, can cause a significant amount of deleted or truncated end products.[4,10,15,16]

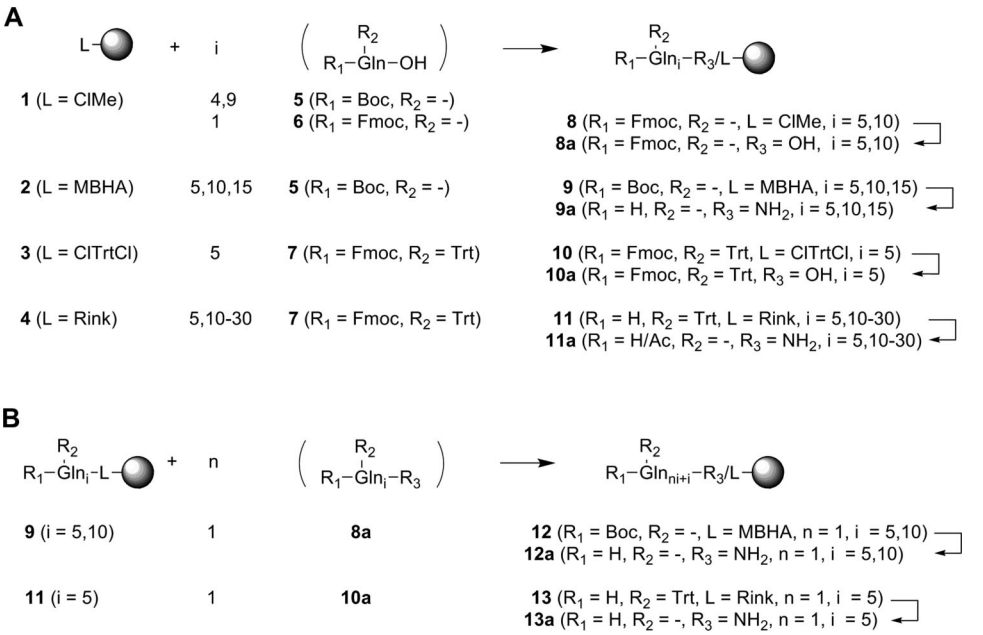
The purification of polyQ peptides also represents a major challenge. The use of highly soluble GST fusion-proteins circumvents the aggregation and solubility problems encountered in the purification steps. However, once the GST tag is cleaved, denatured and filtered out, polyQs are used without further purification.[8,9] The same type of problem is encountered for polyQ peptides produced from solid-phase peptide synthesis once liberated from the solid support. To eliminate the solubility problem, charged amino acids were incorporated at both ends of the glutamine stretch to achieve purification.[2,4,10,15–22] However, charged residues were shown to influence the polyQ aggregation kinetics, the affinity for potential therapeutic agents and the final structure of aggregates.[15,17,23,24]

In this study, two strategies were evaluated to generate uncharged polyQ peptides of various lengths: a linear solid-phase peptide synthesis (LSPPS)[25] and a convergent strategy (CSPPS).[26] Both strategies were evaluated with minimal and maximal side-chain protection schemes and were performed with *t*-butyloxycarbonyl (Boc) and fluorenylmethyloxycarbonyl (Fmoc) peptide chemistries. Uncharged polyQ peptides of various chain lengths were produced with good yield and purity. The solubilization properties of the crude peptides and the conditions for their high performance liquid chromatography (HPLC) purification were investigated to optimize the purity of the final product. A rather simple and straightforward method was then designed to produce polyQ peptides in the Q_{10} to Q_{30} range. The occurrence of *N*-terminal cyclization of glutamine to generate pyroglutamyl peptide derivatives was also examined.

EXPERIMENTAL PROCEDURES

Reagents and Solvents

Fluorenylmethyloxycarbonyl-α-protected-L-glutamine(trityl) (Fmoc-Gln(Trt)), *t*-butyloxycarbonyl (Boc)-α-protected-L-Gln, 4-methylbenzhydrylamine hydrochloride salt resin (MBHA resin, 0.8 mmol/g, 1% divinylbenzene (DVB), 100–200 mesh) as well as the 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucylaminomethyl resin (Rink Amide AM resin, 0.5 mmol/g, 1% divinylbenzene (DVB) and 100–200 mesh) were purchased from ChemImpex International (Wood Dale, IL). Fmoc-α-protected-L-glutamine (Fmoc-Gln) was bought from Advanced Chemtech (Louisville, KY). Chloromethylpolystyrene-divenylbenzene resin (ClMe or Merrifield resin, 1% DVB, 200–400 mesh) was purchased from Bio-Rad (Mississauga, ON) and 2-chlorotrityl chloride resin (ClTrt-Cl resin, 1% DVB, 100–200 mesh) was bought from Calbiochem-Novabiochem Corp. (San Diego, CA). *N*,*N*-Diisopropylethylamine (DIEA) and benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP) were purchased from Matrix Innovation (Montreal, QC). *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), diethylether and acetonitrile (ACN) were obtained from Fisher Scientific (Ottawa, ON), hydrogen fluoride (HF) was from Matheson Gas Products (Ottawa, ON) and biograde trifluoroacetic acid (TFA) was bought from Halocarbon (River Edge, NJ). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP, ≥99%),



SCHEME 1 A: Linear SPPS, B: Convergent SPPS. L is the polystyrene-resin linker, R_1 and R_2 are the *N*-terminal and side-chain protecting groups, R_3 is the *C*-terminal end group, *i* and *n* are integers representing the number of SPPS cycles.

1-methyl-2-pyrrolidinone (NMP) and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Peptide Synthesis and Cleavage

All polyQ peptides were synthesized using a semiautomatic home-designed synthesizer and were performed at room temperature. Two solid-phase synthesis strategies were studied simultaneously: linear and convergent peptide syntheses, named LSPPS and CSPPS, respectively (Scheme 1). Because the LSPPS strategy used Gln residues without side-chain protection (**5**, **6**) during Boc chemistry, it is referred to as the minimal protection scheme while Fmoc chemistry using Gln residues with Trt side-chain protection (**7**) is referred to as the maximal protection scheme. Likewise, the CSPPS strategy using segments without side-chain protection (**8a**), is referred to as the minimal protection scheme while the use of segments with all protected side-chains (**10a**), is referred to as the maximal protection scheme.

Linear Peptide Synthesis. LSPPS strategy consisted in the stepwise introduction of Gln residues to produce uncharged *C*-terminal carboxamide polyQ peptides (**9a**, **11a**) and *C*-terminal free carboxylic segments (**8a**, **10a**) for further use in CSPPS.

Minimal Protection Scheme. Free *C*-terminal carboxylic acid peptides (**8a**) were produced with a Merrifield resin (**1**) while the production of *C*-terminal carboxamide peptides (**9a, 12a**) were obtained with a MBHA resin (**2**). The first Boc-Gln (**5**) was attached on the Merrifield resin with the cesium salt method.[27] The substitution level (0.65 mmol/g) was evaluated with the picric acid method.[28] Coupling cycles were composed of (i) deprotection with 40% (v/v) TFA/DCM for 20 min, (ii) neutralization, accomplished by successive 2-min washings of the resin with DCM, EtOH, 1% (v/v) DIEA/DMF and DMF, (iii) coupling with 3 equiv. of BOP-Gln activated residue (**5**, **6**) and 5 equiv. of DIEA[29] in 10% (v/v) NMP/DMF for 1 h, (iv) removal of excess reagents by DMF (2 × 2 min) and DCM (2 ×2 min) washes followed by (v) ninhydrin testing[30]. Before cleavage, peptide-resins (**8**, **9**, **12**) were washed successively with DCM, EtOH, DCM and ether and dried overnight *in vacuo*. An apparatus from Peptide Institute (Osaka, Japan) was used to cleave peptide-resins using hydrogen fluoride (HF) dried over CoF_3, with ethanethiol and *m*-cresol as scavengers (10 ml reagents/g resin, 8:1:1 v/v). The reaction was carried out for 1 h at 0°C. HF was rapidly evaporated and the resin was washed with diethylether. Crude synthetic peptides (**8a**, **9a**, **12a**) were extracted with TFA and precipitated with diethylether after TFA evaporation.

Maximal Protection Scheme. Free *C*-terminal carboxylic acid segments (**10a**) were produced on a ClTrtCl resin (**3**) while the synthesis of the *C*-terminal carboxamide peptides

(**11a**, **13a**) was accomplished with a Rink AM resin (**4**). The substitution level of the ClTrtCl resin (0.64 mmol/g) was determined through Fmoc absorbance (290 nm) after piperidine treatment of the first anchored amino acid.[31] For these syntheses, the coupling cycle was composed of (i) Fmoc deprotection with a 50% (v/v) piperidine/DMF solution for 10 min, (ii) removal of piperidine by DMF (3 × 2 min) washes, (iii) coupling with 3 equiv. of BOP-activated Fmoc-Gln(Trt) residue (**7**) and 5 equiv. of DIEA[29] in 10% (v/v) NMP/DMF, (iv) excess reagents removal accomplished with DMF (2 × 2 min) and DCM (1 × 2 min) washes followed by (v) ninhydrin testing[30]. Peptide-resins (**10**, **11**, **13**) were submitted to the same treatment as that described under the minimal protection scheme prior to cleavage. The cleavage of the ClTrt-peptide (**10**) was achieved with ten 2-min treatments with a 1% (v/v) TFA/DCM solution. TFA was neutralized by adding a 10% (v/v) DIEA/methanol mixture to the peptide solution followed by a reduction of the solvent volume by evaporation. The remaining peptide fragment (**10a**) was precipitated with water and dried after recovery. Cleavage of the Rink AM-peptide resin (**11**, **13**) was achieved with a 2 h treatment with TFA/phenol/water (20 ml reagents/g resin, 95:2.5:2.5 v/w/v). Peptides were extracted by washing thoroughly the resin with TFA. The crude material (**11a**, **13a**) was recovered by precipitation with diethylether after TFA evaporation.

Convergent Peptide Synthesis. CSPPS strategy was used to evaluate the condensation of the *C*-terminal free carboxylic polyQ segments with a polyQ peptide-resin. Under the minimal protection scheme, the condensation reaction involved *N*-Fmoc polyQ segments synthesized on a Merrifield resin (**8a**) and *N*-deprotected polyQ peptides anchored to a MBHA resin (**9**). As for the maximal protection scheme, *N*-Fmoc-$[Gln(Trt)]_n$ segments synthesized on a ClTrt-Cl resin (**10a**) were condensed with *N*-deprotected polyQ peptides anchored to a Rink AM resin (**11**). Condensation reactions typically consisted of 24-h-coupling steps involving a two fold excess of the BOP-activated polyQ segment to the polyQ peptide-resin. The reaction was followed by ninhydrin testing and the cleavage of peptide-resins was accomplished as described above depending on to the type of resin.

Peptide Solubilization and Purification

Solubility of the crude polyQ peptides in different organic solvents (ACN, benzene, *t*-butanol, DCM, dioxane, DMF, dimethylsulfoxide (DMSO), hexafluoroisopropanol (HFIP), methanol, *i*-propanol, pyridine, tetrahydrofuran) and acids (acetic acid, TFA) was examined. Solubilization in aqueous media was also evaluated after a lyophilization step. Solubility was qualitatively evaluated on small peptide aliquots (2 mg/ml).

Normal-phase purification conditions were evaluated using thin-layer chromatography (TLC) plates coated with silica. A drop of a polyQ peptide solution (2 mg peptide/ml of 1% TFA/DCM) was applied on the plate and eluted with different solvent systems (ACN, DCM, MeOH, *i*-PrOH, 0.2% TFA/DCM, 10% HFIP/ACN, 80% MeOH/ACN, and 80% MeOH/water). Purification by means of reverse-phase HPLC was also examined with an aqueous peptide solution (0.2 mg peptide/ml of 0.2%TFA/0.2%HFIP/H_2O) using a C_4 column (300 Å, 10 μm, 250 mm × 4.6 mm) from Vydac (Hesperia, CA) and a Jupiter C_{18} column (300 Å, 5 μm, 250 mm × 4.6 mm) from Phenomenex (Torrance, CA). Finally, purification was achieved with a Phenomenex Jupiter C_{18} column (300 Å, 15 μm, 250 mm × 21.2 mm) attached to a Varian ProStar (Palo Alto, CA) system equipped with model 215 pumps and a model 320 UV-VIS detector. The peptide solution to be injected onto this system was prepared as follows: after lyophilization in glacial acetic acid, about 200 mg of peptide was dissolved in 2 ml of TFA to which were added 2 ml of HFIP. Then, 10 ml of 0.06% TFA/H_2O were added dropwise and this peptide solution was diluted in 1 l of distilled water. Peptides were eluted with a linear solvent gradient from A (0.2% TFA/H_2O) to B (10% ACN in solvent A). The flow rate was maintained at 10 ml/min and the product was detected at 215 nm.

Peptide purity was evaluated using a Beckman analytical HPLC system (Fullerton, CA) consisting of a model 128 solvent module and a model 168 photo-diode array (PDA) detector. The separation was achieved with a Phenomenex Jupiter C_{18} column (300 Å, 5 μm, 250 mm × 4.6 mm). The flow rate was set at 1.0 ml/min and the elution was performed with an initial 10 min isocratic condition in 0.06% TFA/H_2O followed, over a 15 min period, by a linear gradient from 0 to 30% ACN in 0.06% TFA/H_2O. Homogeneous fractions were analyzed by matrix-assisted laser desorption-ionization/time-of-flight mass spectrometry (MALDI/TOF-MS) on a Voyager DE system from Applied Biosystems (Foster City, CA) using the PerSeptive GRAMS/32 interface. Analyses were carried out with 337 nm light pulses obtained from a nitrogen laser and an α-cyano-4-hydroxycinnamic acid ionization matrix.[32] Each mass spectrum was recorded in linear mode at an accelerating voltage of 25 kV.

Kinetics of the Pyroglutamyl Formation

$HQ_{15}NH_2$ produced by LSPPS under the maximal protection scheme was cleaved and solubilized in aqueous media as

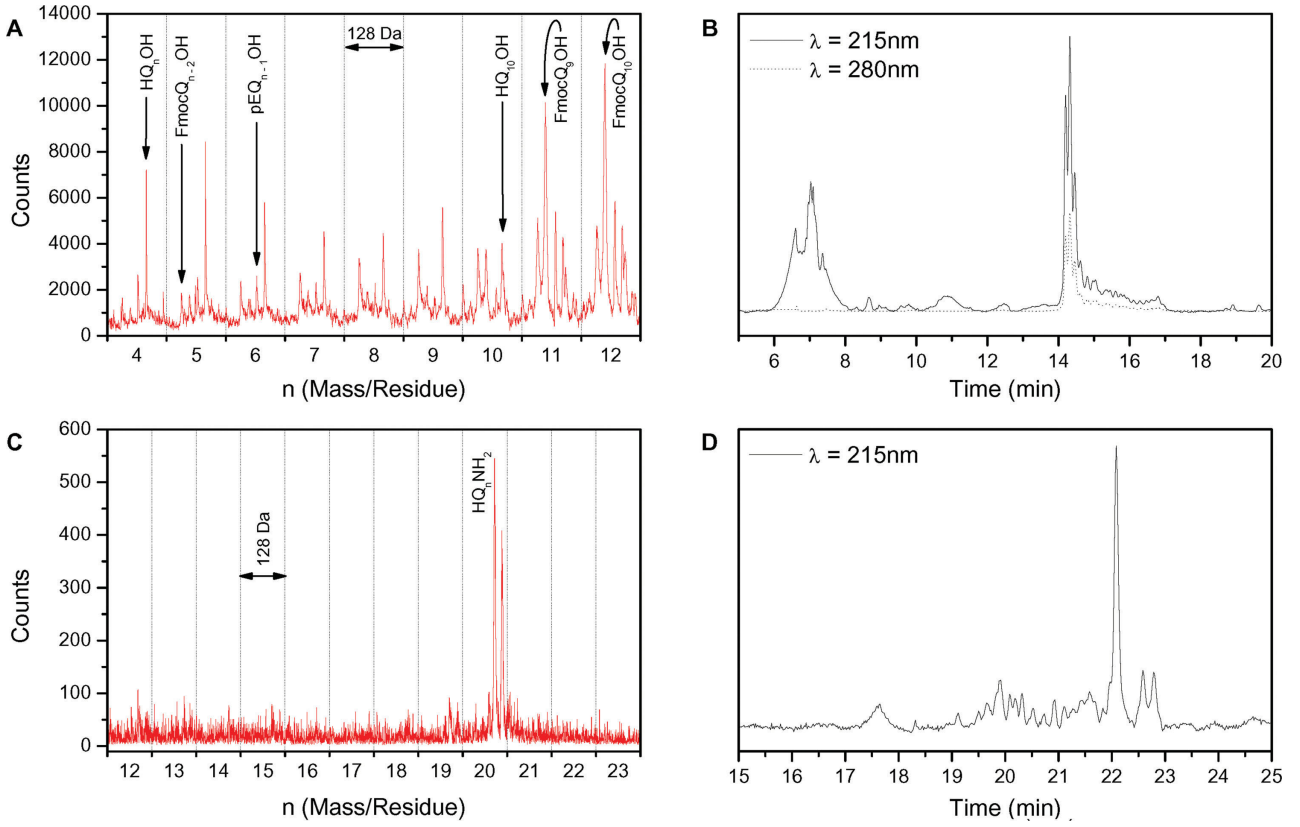


FIGURE 1 MALDI/TOF-MS and analytical chromatograms (HPLC) of crude polyQ peptides produced using LSPPS. A, B: crude $FmocQ_{10}OH$ (**8a**) produced under the minimal protection scheme; C, D: crude $HQ_{20}NH_2$ (**11a**) produced under the maximal protection scheme. M.W. of Gln residue = 128 Da.

described above for purification purposes. A Radiometer PHM61 pH meter (Copenhagen, DK) using an Ag/AgCl glass electrode (Canlab, Montreal, QC) was used to measure pH. The change in peptide concentration was monitored over time using a reverse-phase analytical HPLC, coupled to a photodiode array detector at 215 nm. The first order exponential decay was directly fitted to a Levenberg–Marquardt non-linear regression and analyzed by an unweighted linear regression after data reduction using Microcal Origin 6.0 (Northampton, MA).

RESULTS AND DISCUSSION

Linear Peptide Synthesis

The LSPPS strategy was used to produce *C*-terminal carboxamide polyQ peptides (**9a**, **11a**) and *C*-terminal carboxylic acid segments for further condensation using the CSPPS (**8a**, **10a**). The minimal protection scheme allowed the preparation of *C*-terminal carboxylic acid polyQ segments (**8a**) in good yields (60–70% yields of crude peptide from peptide-resin) but unfortunately a large amount of deletions occurred. Some of the deletions were produced by the incomplete coupling/deprotection as shown by the presence of $FmocQ_nOH$ (n = 2–10) observed on the MS spectrum (Figure 1A) and by the Fmoc absorption at 280 nm ($T_R > 14$ min) recorded on the analytical HPLC chromatogram (Figure 1B). Moreover, products with smaller retention times ($T_R < 14$ min) were observed at 215 nm through analytical HPLC analysis (Figure 1B). These products could correspond to truncated segments resulting from *N*-terminal glutamyl formation since pEQ_nOH (n = 4–9) corresponding masses were found with MS analysis (Figure 1A).[33,34] Furthermore, the presence of a $HQ_{10}OH$ side product showed that some of the *N*-terminal Fmoc protecting groups were removed, probably as a result of the long coupling times and excess reagents (Figure 1).[35] The minimal protection scheme also allowed to prepare *C*-terminal carboxamide polyQ peptides of up to 15 residues (**9a**) although the last five coupling reactions were quite arduous, necessitating increased amounts of BOP-activated residues and long reaction times. Because significant amounts of deletions were observed in the $FmocQ_nOH$

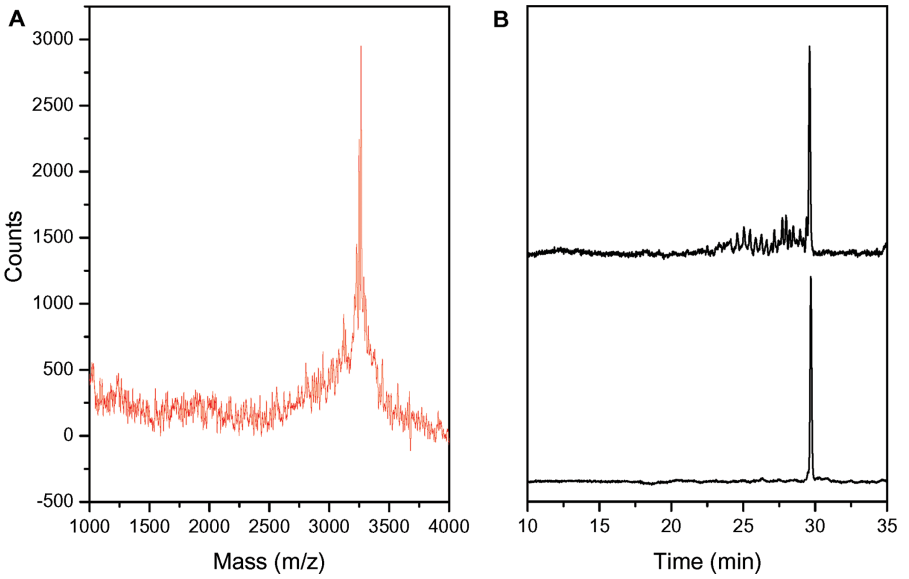


FIGURE 2 A: MALDI/TOF-MS of crude $AcQ_{25}NH_2$ (**11a**) and B: analytical chromatograms (HPLC, 215 nm) before and after purification.

segments and considering the difficulties to achieve coupling reactions, *C*-terminal carboxamide polyQ peptide syntheses under the minimal protection scheme were stopped and peptides (**9a**) were not further characterized. The use of unprotected side-chain Gln residues in this approach probably led to the stabilization of a β-sheet like structure through hydrogen bonding between growing peptide chains, thus creating aggregation related problems.

The LSPPS strategy using the maximal protection scheme was also used to produce *C*-terminal carboxylic acid segments (**10a**) for further use in the CSPPS and *C*-terminal carboxamide polyQ peptides (**11a**). This method allowed the generation of *C*-terminal carboxamide polyQ peptides up to 30 residues (**11a**). Since slow deprotection rates were reported for polyQ synthesis,[4] a more efficient Fmoc removal protocol using 50% piperidine in DMF was used.[36] With this strategy and protection scheme, coupling reactions were carried out easily even after the incorporation of 25 glutamine residues. Moreover, the side-chain protection prevented pyroglutamyl formation and BOP-mediated dehydration of primary amides, which are not reversed by mild cleavage conditions, unlike what occurs under the minimal protection schemes.[37,38] A similar approach using OPfp (pentafluorophenyl) preactivated amino acids had been successfully used by other groups to produce a polyQ sequence with flanking sets of charged amino acids to a sequence.[2,4] Nevertheless, for longer polyQ peptides, Chen and coworkers observed significant levels of deletion.[15,16,20,24] Based on the MALDI/TOF-MS analyses, no such deletions occurred in our peptides (Figures 1C and 2A). Furthermore, this approach allowed the use of a rather high resin loading (0.5 mmol/g) and did not require the use of expensive and somewhat slowly reacting OPfp preactivated amino acids.[39] Yields of crude peptide from peptide-resins were greater than 90%. The results of LSPPS are summarized in Table I.

Convergent Peptide Synthesis

Although CSPPS strategy appeared more promising than LSPPS, because of reduced reaction times and easier side product removal, no polyQ peptides were obtained using this procedure as incomplete couplings provided peptides that differ by five/ten amino acids. In fact, peptide segments synthesized using a minimal protection scheme (**8a**) were rather poorly soluble in all usual coupling media thus preventing any condensation reaction between the fragments. This explains why the yield was not determined (Table I). On the other hand, the use of Trt side-chain protections under

Table I Outcomes of the Different SPPS Strategies and Protection Schemes

Entry	SPPS Strategy	Protection Schemes	Yields (%)[a]	Deletions
9a	Linear	Minimal	60–70	Yes
11a	Linear	Maximal	$\geq$90	No
12a	Convergent	Minimal	N/D	N/D
13a	Convergent	Maximal	$<$10	N/D

[a] Estimations from HPLC analyses of crude products. N/D, Not determined.

the maximal protection scheme permitted ready solubilization of the *N*-α-Fmoc[Gln(Trt)]$_5$-OH fragment (**10a**) in DMF. Although similar conditions had previously allowed the assembly of protected peptide segments to a growing resin-bound peptide to generate portions of proteins associated with Alzheimer's disease,[40] the yields of the coupling reactions that we obtained were poor (estimated <10%) even after using a twofold excess of the BOP-activated peptide segments for two 24-h steps. The low rate of this coupling reaction could be due to the steric effect of the bulky Trt protecting groups linked to the segments. Because we had no success using the CSPPS strategy, the synthesis cycle was not repeated more than once ($n = 1$ in Scheme 1B), thus explaining the undetermined occurrence of segment deletions (Table I). However, if the synthesis cycle had been further repeated, we would have expected to produce a large number of segment deletions. The cleavage of the peptide-resins produced using the minimal protection scheme (**12**, i = 10) gave some of the Fmoc-protected polyQ segment (**8a**) along with the carboxamide peptide (**12a**). The presence of the condensating segment (**8a**) in the resulting crude peptide (**12a**) could be related to a strong interaction between the two peptide chains which would be stable under all washing steps prior to cleavage. Altogether, these observations suggest that the aggregation between unprotected polyQ, in solution or resin-anchored, is a factor leading to the poor coupling and/or deprotection efficiency encountered in both linear and convergent syntheses strategies using the minimal protection scheme.

Peptide Solubilization and Purification

The physico-chemical properties of the glutamine residue, that is the existence of polar and neutral moieties in the molecule, are responsible for the difficulties encountered in solubilizing and purifying polyQ peptides. Indeed, the chemical neutrality of the molecule prevents any straightforward solubilization in aqueous mixtures while its polarity is responsible for intermolecular hydrogen bonds leading to β-sheet aggregation. Because of the lack of charges in polyQ peptides, different organic solvents were tested for their solubilization abilities. Unfortunately, even a solvent such as DMSO, which possesses disaggregating properties and was employed to dissolve polyQ molecules,[18] was unable to solubilize the crude polyQ peptides. Strong organic acids like TFA allowed dissolving and maintaining the peptides in solution even after the addition of organic solvents. As such, mixtures of 1% TFA in DCM or ACN and 2% HFIP/2% DMSO/ACN were appropriate for dissolving the crude peptides at a concentration of 2 mg/ml, although aggregates appeared after a few hours (∼3 h) in TFA/DCM solutions. The addition of HFIP and DMSO to the organic mixture prevented the aggregation for up to 24 h. Because organic acids seem to be a good medium to dissolve polyQ peptides, it could be hypothesized that disaggregation is favoured under these conditions since hydrogen bonds may be disrupted by excess protons. Such structural changes have been observed in ovalbumin with decreasing pH.[41] Indeed, the protein adopts a more flexible molten globule-like state at low pH (2.6–2.8) due to a decrease of the hydrogen bond strength between amide groups. Furthermore, Chehin and coworkers noticed that pH changes could perturb the stability of a lentil lectin, a protein adopting a complete β-sheet structure.[42]

Uncharged polyQ peptides were submitted to the solubilization protocol used for polyQ peptides with charged flanked residues,[20] but addition of water, even in very small quantities, always resulted in peptide precipitation. Thus, the protocol that we designed for producing uncharged polyQ peptides requires a preliminary step consisting in lyophilizing the crude peptide in glacial acetic acid. The peptide was then solubilized in TFA followed by the addition of HFIP before adding water. Such a procedure, allowed the dissolution of all polyQ peptides in aqueous conditions. We hypothesized that glacial acetic acid would break the aggregates, as does TFA, but its advantage is that it can be frozen and lyophilized. Lyophilization would then avoid the concentration of the monomeric species, thus disfavoring the formation of hydrogen bonds between molecules. Moreover, lyophilization would have an additional beneficial effect by removing from the crude material remaining volatile compounds resulting from the TFA cleavage procedure or generated during this step. Therefore, this procedure was found to be very useful and easy to perform on a large scale.

Because solubilization of polyQ peptides was achievable in organic solvents, a normal-phase chromatographic purification strategy was considered. However, the polar character of the glutamine residues caused a strong interaction with the silica stationary-phase. In fact, no migration was observed on TLC plates with DCM, ACN, methanol or with solvent mixtures such as 0.2% TFA in DCM, 10% HFIP in ACN, 80% methanol in ACN and 80% methanol in water. Furthermore, aggregates were observed within 15 min in polyQ peptide solutions prepared with organic solvents, in the absence of DMSO. Although this observation did not seem to interfere with a TLC analysis, it would have been a problem during an HPLC purification step because aggregation prevents any interaction between silica and the peptide.

On the other hand, purification by means of reverse-phase HPLC was not easily achieved because the polarity of the glutamine residues considerably weakened the interaction

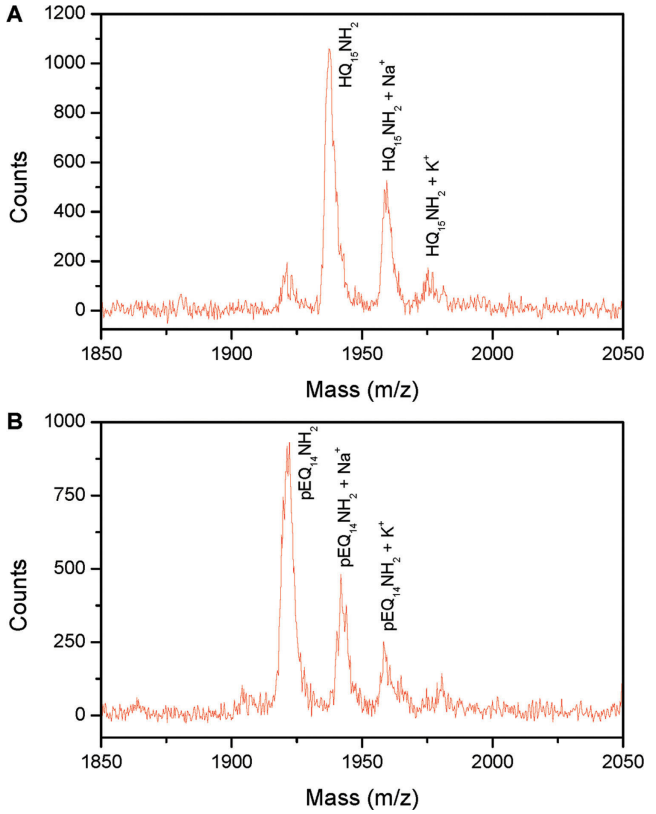


FIGURE 3 MALDI/TOF-MS spectra of $HQ_{15}NH_2$ (**11a**) (A) and $pEQ_{14}NH_2$ (B). The mass difference between those two products is 17 Da.

between the peptides and the bonded-phase. In fact, polyQ peptides were not retained on a C_4 bonded stationary-phase and only weakly adsorbed on a C_{18} matrix. Nevertheless, by reducing the flow rate to 50% of its nominal value (from 20 to 10 ml/min) and by choosing a 2-h linear solvent gradient from A (0.2% TFA/H_2O) to B (10% ACN in solvent A), we were able to attain a very satisfactory separation efficiency and pure peptide preparations were collected. Furthermore, the purification became easier as the number of glutamine residues increased since the retention time correspondingly increased. The purification yield (amount of pure peptide over amount of crude peptide) varied between 20% and 40% for polyQ peptides with a $\geq$95% purity as evaluated by analytical reverse-phase HPLC (Figure 2B).

Pyroglutamyl Formation

During the purification steps, two products were isolated although analytical HPLC analysis of crude peptides revealed only one major peak. MALDI/TOF-MS analysis of the two isolated products indicated that one corresponded to the expected polyQ peptide while the other was a derivative of the peptide, missing 17 Da (Figure 3). Amino-terminal glutamine residues are known to undergo cyclization to form pyroglutamyl derivatives. This side reaction has been shown to occur during SPPS and in pure peptide solutions, with yields varying with pH, temperature and pressure.[43–45] Apart from peptide synthesis where pyroglutamyl formation is favored under weak acidic conditions,[44] glutamine residue cyclization occurs generally at a faster rate in alkaline solutions and under elevated temperature or pressure conditions.[43,45] Interestingly, for our polyQ peptides, this side reaction took place in an aqueous acidic solution (pH 2.5), at room temperature, where the formation of the side product from the crude peptide reached 43% after 24 h, as evaluated by HPLC (Figure 4). The pyroglutamyl formation could have been catalyzed by acetate ions,[33,34] through a mechanism similar to that generating carboxylic acid from glutamic esters.[44] The possible involvement of such catalysts explains why the cyclization is considered a pseudo-first-order kinetic process although the rate of *N*-terminal glutamine degradation follows a monoexponential decay function characteristic of first-order kinetics (Figure 5A).[46] The average rate constant ($\pm$SE) for this cyclization was found to be 66 $\pm$ 1 s^{-1} ($r^2 = 0.98$) for the non-linear fit of the exponential decay and 65 $\pm$ 1 s^{-1} ($r^2 = 0.98$) according to the linear regression of the slope produced by data reduction. The expression of the exponential decay and the non-linear fit using a Neperian logarithm scale allowed to show the excellent agreement between the two slopes obtained from two different analytical treatments. The high dispersion of the residuals indicates that an appropriate model was used to fit the data thus corroborating the first-order kinetics assumption (Figure 5B).[47] As expected, *N*-acetylation of polyQ peptides prevented any degradation of the peptides placed under the same solubilizing conditions, thus supporting the *N*-pyroglutamyl formation postulate. Furthermore, such a modification has been observed in natural biologically active peptides and proteins. Interestingly, *N*-terminal pyroglu-

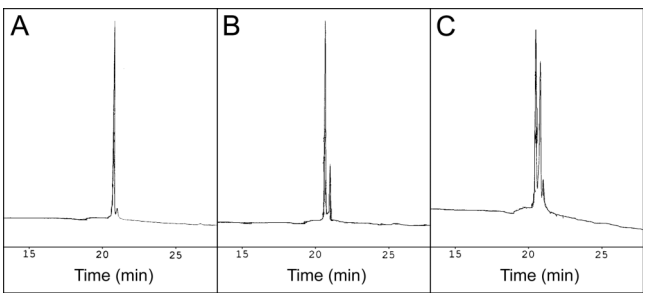


FIGURE 4 Analytical chromatograms (HPLC, 215 nm) showing the Gln to pGlu cyclization. A: pure $HQ_{15}NH_2$ (**11a**), B: after 2.5 h, and C: after 24 h in aqueous solution (pH 2.5). Retention times are 20.6 and 21.0 min for $HQ_{15}NH_2$ and $pEQ_{14}NH_2$, respectively.

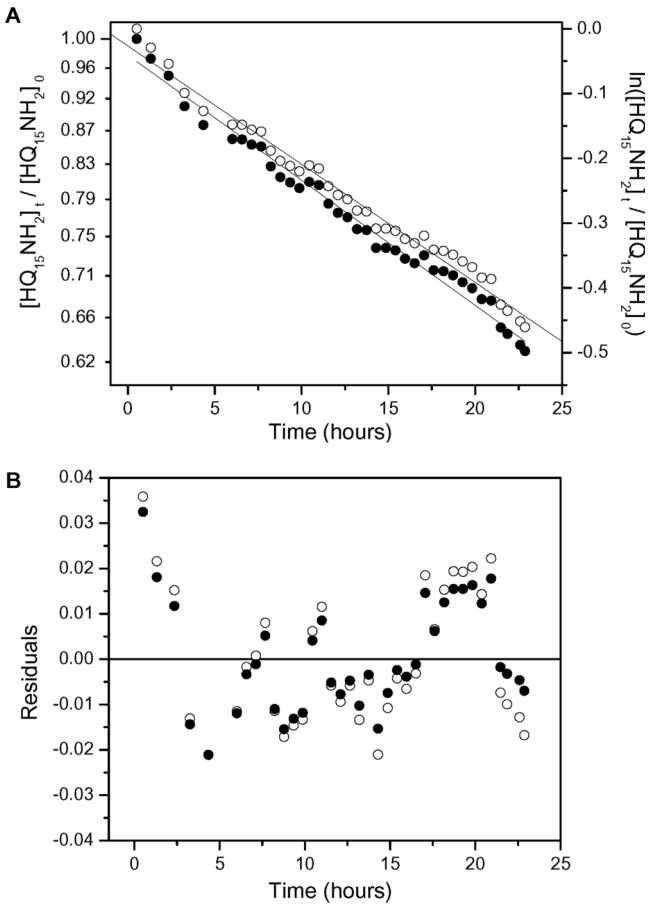


FIGURE 5 A: Pseudo-first-order kinetic graph of pyroglutamyl formation from *N*-terminal glutamine in aqueous solution (pH 2.5). $[HQ_{15}NH_2]_0$ and $[HQ_{15}NH_2]_t$ are the $HQ_{15}NH_2$ (**11a**) concentration at time $t = 0$ and t, respectively; ● mono-exponential decay of $HQ_{15}NH_2$ concentration expressed on a Neperian logarithm scale to facilitate comparison with ○ the linearized counterpart. B: Residual variance plot for both regressions. Points below the line have negative residuals, while points above the line have positive residuals.

tamyl formation has been observed in β-amyloid peptides and the amyloid proteins ABri and Adan, all associated with neurodegenerative diseases.[48,49] Finally, previous hypotheses suggested that such a *N*-terminal modification would boost the polyQ and amyloid peptides related pathogenesis by increasing their catabolic stability, hydrophobicity and aggregation properties, thus leading to enhanced neurotoxicity.[49]

CONCLUSION

Understanding the molecular mechanism by which polyQ fragments intercalate in several proteins and trigger neurodegenerative diseases requires suitable research tools such as charged and uncharged synthetic polyQ peptides. Therefore, a protocol was designed to synthesize and purify glutamine oligomers in good yields and high purity. This goal was attained by coupling Fmoc-Gln(Trt) to a Rink AM resin, and applying a stepwise Fmoc chemistry method. Thereafter, the oligomers of required length were easily cleaved from the solid support under acidic conditions (TFA). The subsequent purification step of the crude peptides was facilitated by a lyophilization step of the material in glacial acetic acid. In fact, this treatment modified the aggregation state of the polyQ peptides and promoted their dissolution in aqueous conditions, thus allowing their purification using reverse-phase HPLC. Interestingly, this step led to the formation of neutral *N*-pyroglutamyl polyQ peptides, a chemical conversion that was shown to happen in proteins responsible for the pathogenesis of some neurodegenerative diseases. Hence, we also isolated this by-product. Thus, charged and uncharged polyQ oligomers, key compounds for studying the physico-chemical process of aggregation that is known to be responsible for neurotoxicity of proteins containing polyQ segments, can be produced using this synthetic strategy.

M.V. is the recipient of a studentship from the CIHR and A.S.D. received financial support from the Faculty of Medicine of the Université de Montréal.

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