

Notes & Tips

## High resolution high-performance liquid chromatography separation of polyglutamic acids

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Poly-L-glutamic acids can exist in random coil,  $\alpha$  helix, or  $\beta$  sheet depending on the environmental conditions. Therefore, they have been used as molecular models for a better understanding of macromolecular stereochemistry [1]. Polyglutamylolation is also the major posttranslational modification of neuronal tubulin [2,3], and foliates are polyglutamylated in most organisms, with pentaglutamates being the predominant forms of foliate polyglutamylolation in a number of species [4]. Poly-L-glutamic acids were also found to be related to a variety of biological functions such as the enhancement of the transgene expression for plasmids [5] and the preferential delivering of paclitaxel [6,7], a widely used anti-cancer agent, to tumor sites, thereby improving its effectiveness and reducing its toxicity.

Polyglutamic acids are highly charged in physiological pH solution. Many of the properties of polyglutamic acids in solution vary considerably with molecular weight. The accurate determination of molecular weights and the molecular weight distribution of polyglutamic acids used in chemical and biochemical experiments are essential. The methods for the estimation of the range of the molecular weight and the molecular weight distribution of polyglutamic acids were mainly based on viscometry and light-scattering determination. An improvement in accuracy was achieved with the introduction of size exclusion chromatography combined with online differential viscometer, differential refractometer, and light-scattering detectors [8].

The separation of oligomers of glutamic acid was also achieved by high-performance liquid chromatography (HPLC) using RPC-5 chromatography [9]. However, RPC-5 chromatography suffered from poor reproduc-

ibility because of its poor pH stability and organic solvent bleeding of the coated Adogen 464, a trialkylmethylammonium chloride, even under normal conditions [10]. The commercial nonavailability of the RPC-5 column also limited the widespread application of the method. Here we present a novel HPLC method for the separation of polyglutamic acids, which gives excellent separation of polyglutamic acids from monomer to 32-mer.

### Materials and methods

The L-amino acids, *N,N'*-carbonyldiimidazole (CDI),<sup>1</sup> L-glutamyl-glutamic acid, and polyglutamic acids (MW 1500–5000) were from Sigma. The successive oligomers of L-glutamic acid were produced by adding the solution of L-glutamic acid (pH 8.0, 2°C) to 100 mM solid CDI. After being vortexed and then held at 2°C for 5 min, the tubes were kept at 20°C in a water bath for 24 h. The samples were analyzed by HPLC (Hitachi L7100 pump with UV-VIS L7420 detector) on Zorbax Bio-Oligo column (6.2 × 80 mm, 5  $\mu$ m) using a NaCl gradient (2–80% B in 60 min; buffer A, 20% acetonitril in 0.02 M NaH<sub>2</sub>PO<sub>4</sub> at pH 7; buffer B, 2 M NaCl in buffer A) at a flow rate of 1 ml/min. The elutions were monitored at 214 nm. Polyglutamic acids were dissolved directly using water and then eluted using the above buffers with a linear gradient of NaCl (6–80% buffer B in 80 min) at a flow rate of 1 ml/min. The usual anion exchange chromatography (Allsphere SAX 5  $\mu$ m, 250 × 4.6) was also tried for comparison using conditions similar to those described above.

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<sup>1</sup> Abbreviations used: CDI, *N,N'*-carbonyldiimidazole; ESI, electrospray ionization.

The 3-mer, 4-mer, 5-mer, and 6-mer of L-glutamic acid were prepared from the oligomerization products of 50 mM L-glutamic acid induced by 100 mM CDI by Bio-Oligo HPLC using an  $\text{NH}_4\text{HCO}_3$  gradient (buffer A, 20% acetonitril in 0.1 M  $\text{NH}_4\text{HCO}_3$  at pH 7.4; buffer B, 20% acetonitril in 1 M  $\text{NH}_4\text{HCO}_3$  at pH 8.3; gradient 2–80% B in 40 min). The fractions were lyophilized and then dialyzed for 8 h using Spectra/Por MWCO 1000. Satisfactory ESI MS spectra were obtained in the  $[\text{M} + \text{H}$  or  $\text{M} + \text{Na}]^+$  and  $[\text{M} - \text{H}]^-$  modes for the 3-mer and 4-mer and in the  $[\text{M} - \text{H}]^-$  mode for the 5-mer and 6-mer; 3-mer of L-Glu: calcd. for  $\text{C}_{15}\text{H}_{23}\text{N}_3\text{O}_{10}$  MW 405, detected 428 $[\text{M} + \text{Na}]^+$ , 450 $[\text{M} + 2\text{Na} - \text{H}]^+$ , 472 $[\text{M} + 3\text{Na} - 2\text{H}]^+$ , 494 $[\text{M} + 4\text{Na} - 3\text{H}]^+$ , 404 $[\text{M} - \text{H}]^-$ , 426 $[\text{M} + \text{Na} - 2\text{H}]^-$ , and 448 $[\text{M} + 2\text{Na} - 3\text{H}]^-$ ; 4-mer of L-Glu: calcd. for  $\text{C}_{20}\text{H}_{30}\text{N}_4\text{O}_{13}$  MW 534, detected 535 $[\text{M} + \text{H}]^+$ , 557 $[\text{M} + \text{Na}]^+$ , 579 $[\text{M} + 2\text{Na} - \text{H}]^+$ , 601 $[\text{M} + 3\text{Na} - 2\text{H}]^+$ , 623 $[\text{M} + 4\text{Na} - 3\text{H}]^+$ , 645 $[\text{M} + 5\text{Na} - 4\text{H}]^+$ , 533 $[\text{M} - \text{H}]^-$ , 555 $[\text{M} + \text{Na} - 2\text{H}]^-$ , and 577 $[\text{M} + 2\text{Na} - 3\text{H}]^-$ ; 5-mer L-Glu: calcd. for  $\text{C}_{25}\text{H}_{37}\text{N}_5\text{O}_{16}$  MW 663, detected 662 $[\text{M} - \text{H}]^-$ ; 6-mer L-Glu: calcd. for  $\text{C}_{30}\text{H}_{44}\text{N}_6\text{O}_{19}$  MW 792, detected 791 $[\text{M} - \text{H}]^-$ .

## Results and discussion

Fig. 1 shows the excellent separation of the oligomers of L-glutamic acid using Zorbax Bio-Oligo chromatography. In reaction of 50 mM L-glutamic acid with 100 mM CDI carried out at 20 °C in aqueous solution, the consecutive oligomers from 3-mer to 11-mer were detected, consistent with previous results [9]. It was interesting to find that by simply decreasing the pH of the buffers to pH 6.0 the clear

resolution of L-glutamic acid monomer and dimers was achieved (Fig. 1 inset) in addition to the reproduction of the molecular distribution of polyglutamic acids from 3-mer to 11-mer. Decreasing the pH of the buffers to pH 5 gives even better separation of the short oligomers. The assignment of the polyglutamic acids was based on the coinjection of the authentic samples of L-glutamic acid and glutamyl-glutamic acid, and the ESI MS identified 3-mer, 4-mer, 5-mer, and 6-mer of L-glutamic acid separately.

The longer oligomers of L-glutamic acid were also resolved using Zorbax Bio-Oligo chromatography under the same condition (Fig. 2). Both the 5-mer and the 6-mer identified by ESI MS were coinjected for the assignment of the oligomers. It is clear from Fig. 2 that the commercial polyglutamic acids (Sigma P1818) are actually composed

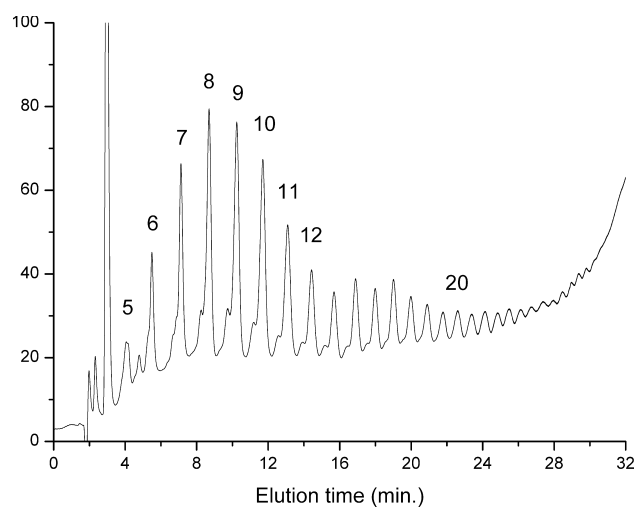


Fig. 2. HPLC elution profile of polyglutamic acids (Sigma P1818).

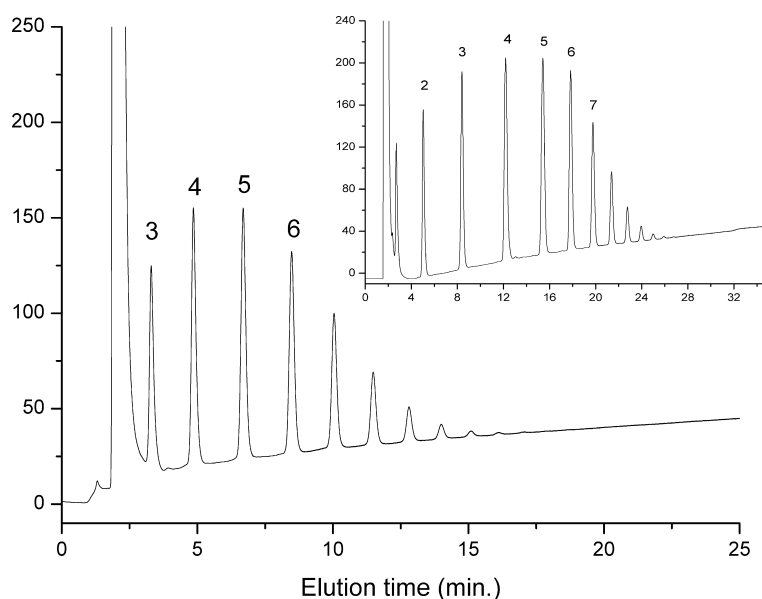


Fig. 1. HPLC elution profile of the oligomers of 50 mM L-glutamic acid induced by 100 mM CDI in aqueous solution (pH 8) from 3-mers and from L-glutamic acid monomer (inset).

of successive oligomers from 5-mer to 32-mer, different from the assigned range of molecular weight. This HPLC profile also clearly demonstrated both the molecular weight distribution of the polyglutamic acids and the presence of significant proportion of isomers of polyglutamic acids.

We demonstrated here that Bio-Oligo chromatography could give convenient and frank separation of both the molecular weight range and the molecular weight distribution of polyglutamic acids from monomers to 32-mers, therefore satisfying the demands for the analysis of polyglutamic acids in known biological polyglutamylation. The highly efficient separation of the oligomers of L-aspartic acid and nucleic acid derivatives by Bio-Oligo chromatography achieved in this study indicates the potential of the Bio-Oligo chromatography in the separation of the other negative-charged molecules.

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