

Expression of compstatin in *Escherichia coli*: Incorporation of unnatural amino acids enhances its activity

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Abstract

Compstatin, a 13-residue cyclic peptide, is a complement inhibitor that shows therapeutic potential. Several previous approaches have improved the activity of this peptide several-fold. In the present study, we have expressed and purified compstatin from *Escherichia coli* in an effort to increase its potency and to generate it in high yield in a more economical fashion. An intein-based expression system was used to express compstatin in fusion with chitin-binding domain and *Ssp* DnaB intein, which were later cleaved from the expressed molecule at room temperature and pH 7.0 to yield pure compstatin in one step. The expressed compstatin showed activity similar to the synthetic compstatin in an ELISA-based assay. The same expression system and purification strategy were used to incorporate three tryptophan analogs, 6-fluoro-tryptophan, 5-hydroxy-tryptophan, and 7-aza-tryptophan, into compstatin. Interestingly, incorporation of 6-fluoro-tryptophan increased the activity three-fold relative to wild-type compstatin; in contrast, incorporation of 5-hydroxy- or 7-aza-tryptophan rendered compstatin less active than the wild-type form.

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Compstatin is a 13-residue cyclic peptide inhibitor that binds to the third component of complement and abrogates the activation of complement cascade [1–7]. Compstatin, as shown using NMR, folds into a loop-like structure with a type I beta turn in solution and remains cyclic through a disulfide bond bridging residues Cys-2 and Cys-12 [8]. This peptide was originally discovered by screening a phage-displayed peptide library; all the subsequent design and development were carried out using the peptides that had been chemically synthesized employing solid-phase peptide chemistry [1,9]. The drawbacks of this solid-phase peptide synthesis approach include its high cost and the possible presence of residual chemicals that can interfere with the biological activity.

The goal of the present study was to express compstatin in bacteria using an intein-based vector and subsequently incorporate tryptophan analogs into the peptide, since substituting tryptophan for valine at position 4 has been shown to increase the activity of compstatin several-fold [7]. Since most of the tryptophan analogs are available currently as a mixture of D and L forms, solid-phase peptide synthesis becomes cumbersome, as the peptides containing D and L forms of tryptophan (analogues) have to be separated. Previous studies carried out with compstatin analogs containing D-amino acids have shown no activity for these peptides. Hence, only peptides containing L-amino acids are desired, and separation of peptides with L-tryptophan analogs from the mixture results in lower yields. Therefore, we have attempted to express compstatin in *Escherichia coli* to increase the yield in a more economical way, since *E. coli* is capable of incorporating only L-amino acids, and thus provides a useful system for efficiently incorporating amino acids even when present as a DL mixture [10–13].

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In the present study, we have used an intein-based expression system to express compstatin. Inteins have a unique capacity to cleave themselves from exteins in the process of generating a functional protein. This property has been exploited by Xu and co-workers [14] to design expression systems in which the protein of interest is fused with an intein and later cleaved under defined conditions that support intein cleavage. This expression system is cost-effective, as the protein of interest can be purified in a single step, and no enzyme cleavage is required to obtain the final product. As a result, this system has been widely employed to address several applications involving proteins and peptides [14–23]. Using this expression system, we succeeded to express compstatin and its analogs, some of which showed increased activity.

Experimental procedures

Reagents

The pTWIN 1 vector was obtained from New England Biolabs (Beverly, MA, USA). SOB medium, sodium phosphate, sodium chloride (NaCl),¹ ethylenediamine tetraacetic acid (EDTA), ammonium acetate, isopropyl-beta-D-thiogalactopyranoside (IPTG), M9 minimal medium, 6-fluoro-DL-tryptophan, 7-aza-DL-tryptophan, and 5-hydroxy-L-tryptophan were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Construction of the compstatin gene

A compstatin analog with the sequence NH₂-GIC-VWQDWGAHRCTN-OH, designated [G(-1)/V4W/H9A/N14], was expressed in fusion with the chitin-binding domain (CBD) and the DnaB intein (New England Biolabs). Back-translation of the peptide sequence, taking into consideration the codon usage for *E. coli*, provided the following genetic code, which was used to generate a synthetic gene for this peptide with the sequence:

5'-ATTTGCGTTTGGCAGGATTGGGGTGCACCGTTGCACCAATTA-3'.

To clone the synthetic gene into the pGEM-T vector, a 5' flanking region containing a *SapI* site and 3' flanking region containing a *PstI* site were designed. To construct the synthetic gene, the four overlapping oligonucleotides shown below were designed using DnaWorks software [24] and synthesized at Invitrogen (Carlsbad, CA, USA):

5'-GGTGGTGCTCTTCCAACGGTATTTGCGTTTGGCAGGA-3'

5'-TTGGGGTGCACCGTTGCACCAATTAAGCAGG-3'

3'-CAACGTGGTTAATTGACGTCCGC-5'

3'-CATAAACGCAAACCGTCCTAACCCACGCGTGG-5'.

The overlapping DNA fragments were assembled by PCR as described by Heyneker and co-workers [25].

Cloning of the compstatin gene

The resulting gene was amplified using the primers 5'-CGCTGCAGTTAATTGGT-3' and 5'-GGTGGTGCTCTTCCAACG-3'.

The PCR-amplified fragments of compstatin were then cloned into the pGEM-T vector, and the resulting clone was digested with *PstI* and *SapI*. The digested compstatin gene was further subcloned into the expression vector pTWIN1, which had been predigested with *PstI* and *SapI*. The sequence of the clone was verified at the DNA Sequencing Facility of the University of Pennsylvania. Fig. 1 shows the vector construct depicting different genetic elements.

Expression and purification of compstatin

To express compstatin, ER2566 *E. coli* cells transformed with the compstatin clone were grown in SOB medium (20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 2.5 mM KCl, and 10 mM MgCl₂) at 37°C. When an OD₆₀₀ of 0.7 was reached, expression was induced by the addition of IPTG to a final concentration of 0.3 mM, followed by an additional incubation at 37°C for 4 h. Cells were collected by centrifugation and lysed by sonication in 20 mM phosphate buffer, pH 8.5, with 500 mM NaCl and 1 mM EDTA (buffer A) supplemented with 0.2% Tween 20. The cell extract was centrifuged, and the soluble fraction was applied to a chitin-binding column pre-equilibrated with buffer A. The insoluble pellet was solubilized using buffer A containing 8 M urea, and after diluting 10 times using buffer A was applied to the column. The column was washed with 100 mL of buffer B1, followed by a quick wash with three column volumes of 50 mM ammonium acetate, pH 7.0 (buffer B). The column was incubated at room temperature for 20 h, and the peptide was eluted with buffer B, lyophilized, and further purified on a reversed-phase C18 high-performance liquid chromatography (HPLC) column using a 0–90% acetonitrile gradient. The purified peptide was identified and characterized using matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF) mass spectrometry.

Expression of tryptophan analogs of compstatin in *Escherichia coli*

To express compstatin analogs containing tryptophan derivatives, the pTWIN1-compstatin clone was transformed into the ER2566 Trp 82 auxotroph (obtained from Bjorklund) [23]. Expression was carried out in M9 minimal medium supplemented with 1 mM L-tryptophan as described above.

¹ Abbreviations used: Ac, acetyl group; OH, hydroxy; 6fW, 6-fluoro-tryptophan; NaCl, sodium chloride; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; CBD, chitin-binding domain; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; HPLC, high-performance liquid chromatography; PHMB, *para*-hydroxyl mercury benzoate.

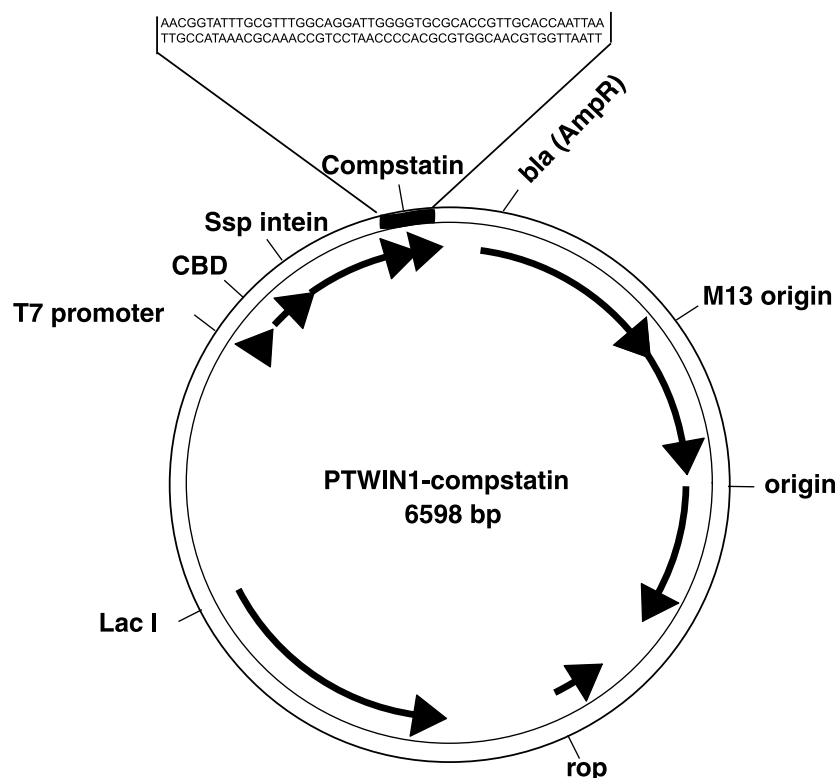


Fig. 1. Construction of the compstatin gene. A schematic showing the location of compstatin insert in the pTWIN1 vector. Also shown are the locations of the other genetic elements that assist in the replication and expression of the plasmid. This picture is drawn using the ApE-plasmid editor software (<http://www.biology.utah.edu/jorgensen/wayned/apE>).

Cells were grown to an OD_{600} of 0.8–1.0, then collected by centrifugation, and resuspended in fresh minimal medium containing 2 mM of the desired tryptophan analog(s): 6-fluoro-tryptophan, 7-aza-tryptophan, or 5-hydroxy-tryptophan. The expressed compstatin analogs were further purified as described above.

Activity measurements for expressed compstatin

The complement inhibitory activity of compstatin analogs was assayed as described elsewhere [7]. In brief, an antigen–antibody complex was coated onto an ELISA plate, and the deposition of C3b in the presence of the compstatin analog was measured. The absorbance data obtained at 405 nm were translated into % inhibition based on the absorbance corresponding to 100% complement activation. The % inhibition was plotted against the peptide concentration, and the resulting dataset was fit to the logistic dose–response function using Origin 7.0 software. IC_{50} values were obtained from the fitted parameters that achieved the lowest χ^2 value.

Results and discussion

Construction and cloning of the gene

The gene encoding compstatin, together with the flanking restriction enzyme sites, is 74 nucleotides long. The gene was assembled using short overlapping fragments encom-

passing the entire 74 nucleotides. Upon cloning into the pTWIN1 vector, the *SapI* site was lost, while the *PstI* site was regenerated. Flanking the multiple cloning site in this vector is a region that encodes an intein, *Ssp* DnaB fused with the CBD at its N-terminus. While the CBD facilitates the isolation of the fusion protein from the cell extract, the intein activates self-cleavage and releases compstatin free of any added (extra) amino acids. Fig. 1 shows the final construct depicting the sequence of the insert and other genetic elements that contribute to the expression. The sequence of the clone was verified using the *Ssp* DnaB intein forward primer.

Expression and purification of compstatin

The fusion protein CBD-DnaB-compstatin was purified from the soluble fraction of cell lysate on a chitin column followed by a reverse-phase C18 column; the final yield was 2 mg/L of the culture. Fig. 2 (lanes 2 and 3) shows a 13% SDS-PAGE gel of compstatin in fusion with *Ssp* DnaB–CBD. The peptide was further characterized using MALDI-TOF spectrometry and HPLC; single peaks (Fig. 4) in each of these profiles indicate that the peptide was obtained in pure form.

To incorporate tryptophan analogs into compstatin, the clone bearing the compstatin gene was transformed into a tryptophan-auxotrophic ER2566 strain. Previous studies have indicated that *E. coli* is capable of incorporating these

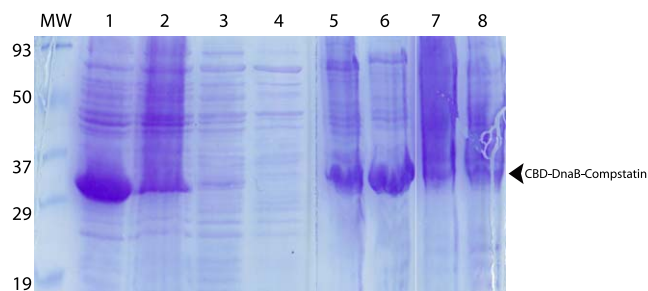


Fig. 2. SDS-PAGE analysis of expressed compstatin-fusion proteins. A 13% SDS-PAGE gel showing the induction of the compstatin-fusion gene expression by 0.3 mM IPTG at 30 °C in the presence of tryptophan (lanes 1 and 5), 6-fluoro-tryptophan (lanes 2 and 6), 5-hydroxy-tryptophan (lanes 3 and 7), and 7-aza-tryptophan (lanes 4 and 8). Lanes 1–4 represent soluble fraction, while lanes 5–8 represent insoluble cell pellet solubilized in 8 M urea. Molecular weights of the standards are expressed in kilodalton.

analogs without modifying its translation machinery [11,26–33]. Following induction with IPTG, the fusion protein containing tryptophan resulted in the soluble fraction (lane 1). In contrast, the fusion protein with 6-fluoro-tryptophan incorporated instead of tryptophan expressed in lower levels and resulted in inclusion bodies as shown in Fig. 2 (lane 2, soluble; lane 6, insoluble). This distinction could have occurred as a result of 6-fluoro-tryptophan rendering the fusion protein more hydrophobic compared to tryptophan. In the case of 5-hydroxy-tryptophan and 7-aza-tryptophan incorporation, due to the low expression levels, we were unable to assess if the fusion protein is localized in soluble or insoluble fraction (Fig. 2). Following single-step purification, pure compstatin was obtained with the yields of 1.2 mg/L for 6-fluoro-tryptophan, 0.2 mg/L for 5-hydroxy-tryptophan, and 0.05 mg/L for 7-aza-tryptophan. The low yield for these peptides can be attributed to poor growth of cells in the presence of tryptophan analogs. As shown in Fig. 3, in the presence of tryptophan analogs, 6-fluoro-tryptophan

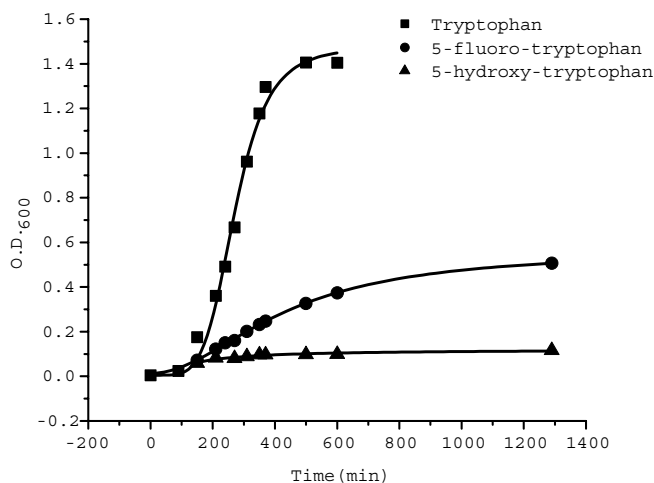


Fig. 3. Growth curves of the Trp-82 auxotrophic ER2566 strain. The plot shows the optical density of the cells grown in the presence of tryptophan (squares), 6-fluoro-tryptophan (circles), and 5-hydroxy-tryptophan (triangles) as a function of time.

and 5-hydroxy-tryptophan, *E. coli* exhibits slower growth while in the presence of 7-aza-tryptophan, the growth was minimal. The incorporation of tryptophan analogs was confirmed using MALDI as indicated by the spectra shown in the inset of Figs. 4 and 5 and the molecular masses are listed in Table 1. Both native compstatin and its analogs in the final pure forms were shown to be cyclized through a disulfide bond, as evidenced by their inability to react with *para*-hydroxyl mercuryl benzoate (PHMB) [34] (data not shown). The cyclization could have occurred naturally during the process of purification.

All the peptides were further purified on a reversed-phase C18 HPLC column. For each of the peptides containing 6-fluoro-tryptophan and 5-hydroxy-tryptophan, three peaks were observed in the chromatogram (Fig. 5). The sequence of expressed compstatin contains two tryptophans at positions 4 and 7 and ideally single cyclized species of compstatin containing tryptophan analog at both the positions is expected. However, if the medium is contaminated with tryptophan through a carry over from the media exchange during the induction, peptides with tryptophan incorporated simultaneously at both the positions or at either of the positions are also expressed. The mass identification of the peptides (Table 1; Fig. 5) eluted in each of the three peaks observed in the chromatograms using MALDI indicated that differential incorporation of the tryptophan analogs occurred. None of these peptides represent the linear non-cyclized compstatin as evidenced from the masses obtained from the MALDI spectra. In addition, the mass spectra of the peptides representing each of these peaks indicated that the peptides were eluted in increasing order of the degree of incorporation of the fluoro analog. In contrast, the elution order was reversed in the case of peptides containing 5-hydroxy-tryptophan. These differential changes in the elution order reflect the relative hydrophobicity of the peptides altered as a result of the incorporation of the tryptophan analogs. For example, 5-hydroxy-tryptophan is hydrophilic, while 6-fluoro-tryptophan is hydrophobic.

Activity of expressed compstatin and its analogs

The expressed compstatin [G(-1)/V4W/H9A/N14] exhibited an IC_{50} of 1.2 μ M, which is similar to that observed for the synthetic Ac-V4W/H9A analog [7]. It was previously suggested that acetylation neutralizes the charge on the N-terminus of compstatin, thus preserving the hydrophobic cluster required for the activity [35]. Our findings in the present study indicate that glycine contributes in preserving the hydrophobic cluster, thus eliminating the need for acetylation. All the expressed compstatin analogs except the 7-aza-tryptophan analog were found to be active at the concentrations tested. However, the peptide showed different levels of activity relative to the synthetic analog, Ac-V4W/H9A (Fig. 6; Table 2): compstatin containing 6-fluoro-tryptophan exhibited a three-fold higher activity than did the Ac-V4W/H9A analog, suggesting that increasing the hydrophobicity of the indole ring by adding fluorine atom increases the activity of the peptide (Fig. 6).

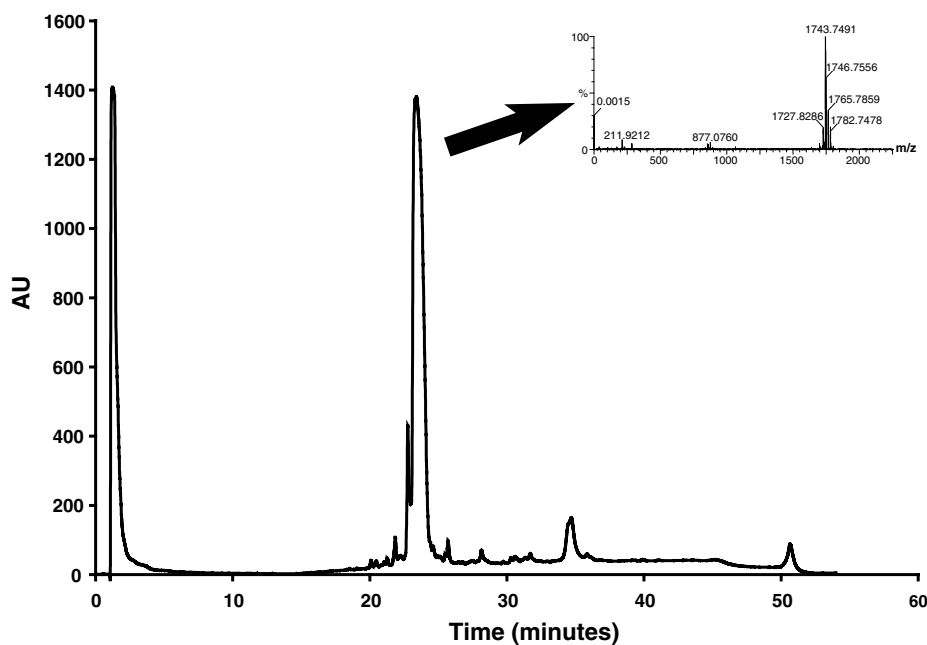


Fig. 4. Purification of expressed compstatin. Compstatin was expressed using an intein-mediated expression system and purified using reversed-phase HPLC employing a linear gradient of 5–90% acetonitrile. An HPLC chromatogram representing purified compstatin is shown. A MALDI spectrum of the purified compstatin is shown in the inset. The m/z ratio represents the masses of the protonated species.

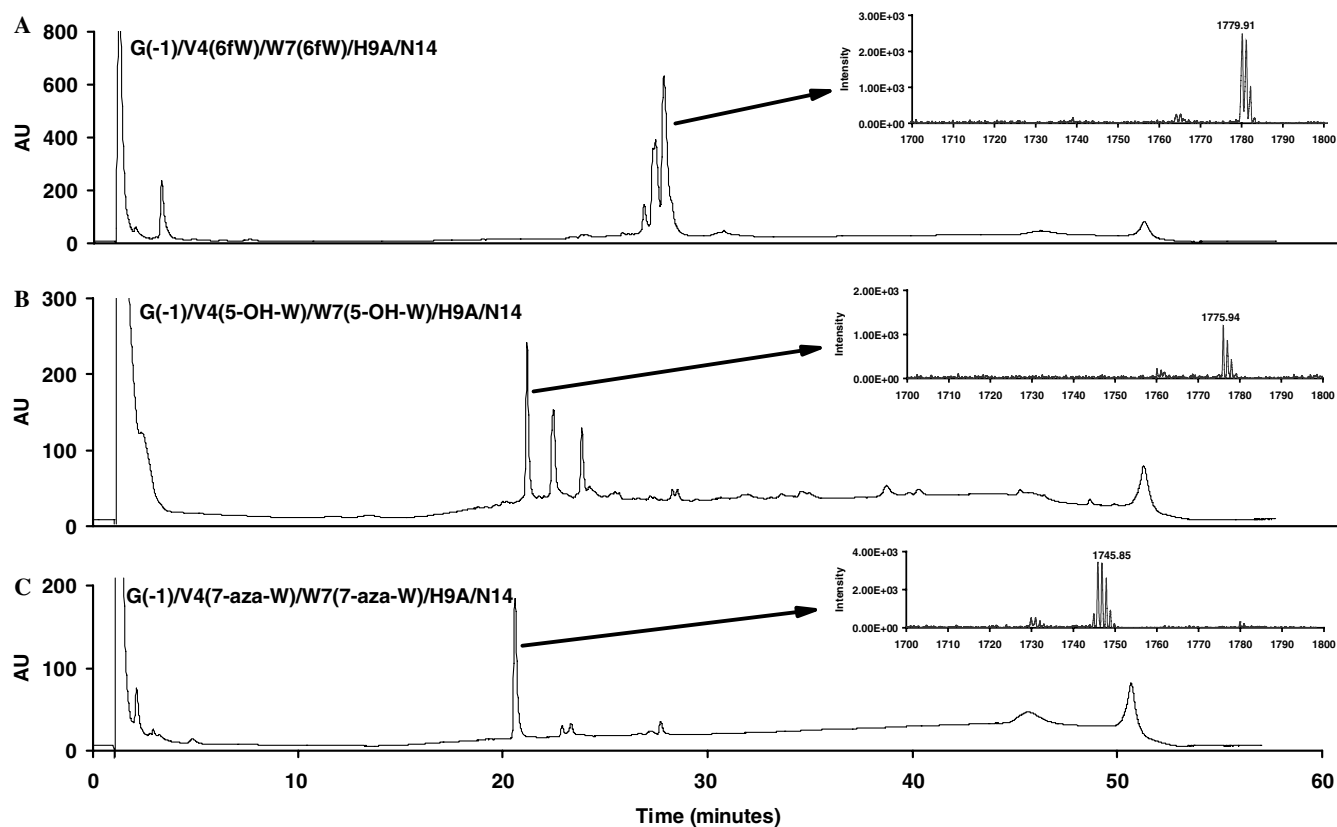


Fig. 5. Characterization of compstatin analogs. Shown in the picture are HPLC chromatograms of the expressed compstatin analogs containing (A) 6-fluoro-tryptophan, (B) 5-hydroxy-tryptophan, and (C) 7-aza-tryptophan. The corresponding mass spectra for the fully incorporated compstatin analogs are shown in the inset. The m/z ratio represents the masses of the protonated species.

We also investigated the incorporation of the less hydrophobic tryptophan analogs 5-hydroxy-tryptophan and 7-aza-tryptophan as a means of assessing the significance of

the hydrophobic nature of the indole ring in the interaction of compstatin with C3. In contrast to the results obtained with the 6-fluoro analog, the compstatin analog containing

Table 1
Molecular masses of compstatin and its analogs

Expressed peptide	Expected molecular weight	Observed molecular weight
G(-1)/V4W/H9A/N14	1743.7406	1743.74
G(-1)/V4(6fW)/W7(6fW)/H9A/N14	1779.7217	1779.91
G(-1)/V4(5-OH ^a -W)/W7(5-OH-W)/H9A/N14	1775.7148	1775.94
G(-1)/V4(7-aza-W)/W7(7-aza-W)/H9A/N14	1745.7311	1745.85

^a Represents hydroxy.

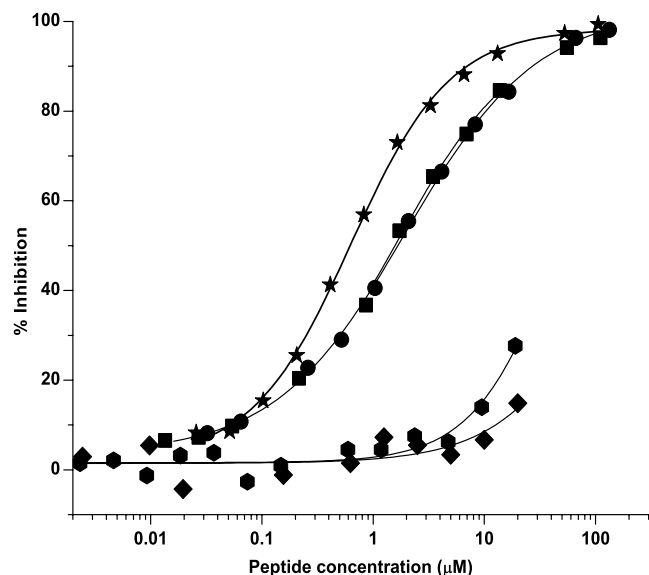


Fig. 6. Activity of expressed compstatin and its analogs. Plots of percent complement inhibition versus peptide concentration for Ac-V4W/H9A (squares) and expressed compstatin with tryptophan (circles), 6-fluoro-tryptophan (stars), 5-hydroxy-tryptophan (hexagons), and 7-aza-tryptophan (diamonds).

Table 2
Complement inhibitory activity of the expressed peptides

Peptide	IC ₅₀ (µM)	Relative activity ^a
Ac-V4W/H9A ^b	1.2	45
G(-1)/V4W/H9A/N14	1.2	45
G(-1)/V4(6fW)/W7(6fW)/H9A/N14	0.43	126
G(-1)/V4(5-OH ^c -W)/W7(5-OH-W)/H9A/N14	33	1.6
G(-1)/V4(7-aza-W)/W7(7-aza-W)/H9A/N14	122	0.44

^a Relative to the activity of the peptide I(CVVQDWGHHRC)T-NH₂.

^b Synthetic peptide.

^c Represents hydroxy.

5-hydroxy-tryptophan showed a 28-fold loss in activity when compared to the Ac-V4W/H9A analog, and the peptide containing 7-aza-tryptophan showed no activity at all at any of the concentrations tested. These results indicate that lowering the hydrophobicity of the indole ring results in a loss of activity (Fig. 6). The 7-aza-tryptophan resembles tryptophan in molecular structure, except that it has nitrogen instead of a carbon atom at position 7 of the indole ring. The loss in activity observed after substitution

with 7-aza-tryptophan shows the relative importance of this carbon atom. One possible explanation is that the nitrogen at position 7 of the indole ring reduces the hydrophobic character of the tryptophan. These data suggest that the degree of hydrophobicity of the indole ring influences the activity of compstatin.

In conclusion, fully active compstatin was obtained by expression in *E. coli*, with an yield of 2 mg/L of culture. Incorporation of unnatural amino acids with varying degrees of hydrophobicity at positions 4 and 7 led to a 126-fold increase in activity in the case of the 6-fluoro analog and suggest that increasing the hydrophobicity at these two positions can increase the activity of compstatin.

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