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ORIGINAL ARTICLE

D-Ser-containing humanin shows promotion of fibril formation

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Abstract Humanin (HN), a peptide of 24 amino acid residues, suppresses the neuronal cell death that is induced by the gene products of Alzheimer's disease. HN contains two Ser residues at positions 7 and 14. Because the proportion of D-Ser isomerized from L-Ser in proteins appears to increase as cellular organs age, we explored the structural effects of the isomerization of each Ser residue in HN. By using a thioflavin-T assay to detect fibril formation, we found that an HN derivative that contained two isomerized D-Ser residues had a greater tendency to form fibrils than did wild-type HN or HNs containing single D-Ser residues. A previous report showed that HN containing two D-Ser residues exerts neuroprotective activity. Our data, therefore, suggest that the fibril formation by HN that contains two D-Ser residues may promote HN neuroprotective activity.

Keywords Humanin · D-Ser · Circular dichroism · β-Sheet · α-Helix

Introduction

Although it has long been believed that only L-enantiomers of amino acids occur in nature, the development of analytical methods has disclosed the presence of D-amino acids in vertebrates and invertebrates (D'Aniello et al. 1995; Okuma et al. 1995), both in free form and as components of proteins. Because proteins biosynthesized via ribosome are formed with only L-amino acids, the incorporation of D-amino acids into proteins or peptides occurs by posttranslational modification. In vivo, L-amino acids are replaced with D-amino acids as a result of either enzyme-catalyzed isomerization or spontaneous reactions (Heck et al. 1996; Fujii et al. 1999). Racemization causes some proteins to lose biological activities through their conformational changes, whereas others gain high biological activities. Humanin (HN) is one of the proteins that shows enhancement of biological activities as a result of racemization (Terashita et al. 2003).

HN was identified as a neuroprotective gene by using death-trap screening with a cDNA library from the occipital lobe of a patient with Alzheimer's disease (AD) (Hashimoto et al. 2001c). This peptide comprises 24 amino acid residues, MAPRGFSCLLLLTSEIDLPVKRRA, and inhibits the neuronal cell death caused by various familial Alzheimer's disease-related insults (Hashimoto et al. 2001a, b, c). Because HN is secreted extracellularly, its neuroprotective activity is confined mainly to the outside of the cell via its cell-surface receptor (Hashimoto et al. 2005). Interestingly, substitution of L-Ser at position 14 in HN with D-Ser, [D-Ser^{14}]HN, enhances the neuroprotective function to three orders of magnitude higher than that of HN, whereas phosphorylation of this L-Ser residue does not affect HN activity (Terashita et al. 2003), suggesting that isomerization of the L-Ser residue in HN is important for HN function.

Substitution of each amino acid residue in HN changes the neuroprotective activity of HN. Substitution of Gly for Ser^{14} (S14G-HN; HNG) dramatically increases the

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neuroprotective effect to the same level as that of [D-Ser^{14}]HN, whereas substitution of Ala for Cys8 (C8A-HN; HNA) abolishes the effect because the molecule can no longer self-dimerize (Hashimoto et al. 2001c; Terashita et al. 2003). Recently, several groups elucidated the structural features of HN, HNG, and HNA under various conditions (Arakawa et al. 2006; Arisaka et al. 2008; Benaki et al. 2005, 2006; Pistolesi et al. 2009). However, the structural characteristics of D-Ser-containing HNs, such as [D-Ser^{7}]HN, [D-Ser^{14}]HN, and [D-Ser^{7,14}]HN, have not been defined. Here, we report the secondary structure of HNs containing D-amino acids, as revealed by using circular dichroism (CD) spectroscopy. Furthermore, to study the fibrillization of HNs containing D-Ser, we performed a thioflavin-T (Th-T) assay with fluorescence spectroscopy and a Congo red binding assay.

Materials and methods

Peptides

The following peptides were chemically synthesized by and purchased from Scram (Tokyo, Japan): HN (MAPRGF SCLLLLTSEIDLPVKRRA), [D-Ser^{7}]HN (MAPRGF-**D-S**-CLLLLTSEIDLPVKRRA), [D-Ser^{14}]HN (MAPRGFSCLL LLT- **D-S**-EIDLPVKRRA), [D-Ser^{7,14}]HN (MAPRGF-**D-S**-CLLLLT-**D-S**-EIDLPVKRRA), and HNA (MAPRGFSAL LLLTSEIDLPVKRRA). The Aβ1-42 peptide was purchased from AnyGen (Gwang-ju, South Korea).

CD spectroscopy

HN or its derivatives were dissolved in PBS to a final concentration of 20 μM. In the experiments with 2,2,2-trifluoroethanol (TFE) (Tokyo Chemical Industry; Tokyo, Japan), each HN peptide was diluted in 40% TFE/PBS to a final concentration of 20 μM. CD spectra (wavelength range: 250–195 nm) were measured using a Jasco 820 spectrometer (Jasco; Tokyo, Japan) with a quartz glass cuvette at a 1-mm path length and 0.1-nm intervals at 100 nm/min. Data are shown as mean residue ellipticities. The relative proportions of secondary structure were estimated from the data using the Jasco analytical program (Yang et al. 1986). Data were analyzed statistically by using Student's *t* test.

Thioflavin-T assay

HN or each derivative was dissolved in PBS or 40% TFE/PBS to a final concentration of 20 μM and incubated at 37°C for 2 h. Each HN solution (4 μL) was added to 800 μL of 5 μM Th-T (Wako, Osaka, Japan) in 50 mM Gly–NaOH (pH 8.5). The fluorescence intensity was then measured at 450 nm excitation and 480 nm emission by using a Shimazu RF5300PC spectrofluorophotometer (Shimazu, Kyoto, Japan), as reported previously (Naiki et al. 1989). Data were analyzed statistically by using one-way ANOVA followed by Tukey–Kramer post hoc test.

Congo red binding assay

The Congo red binding assay was performed as described previously (Klunk et al. 1989). Briefly, Congo red (TCI, Tokyo, Japan) was dissolved in PBS and filtered. The absorbance of Congo red solution was measured at a wavelength of 498 nm to determine the concentration. HN or each derivative, dissolved in PBS, was diluted in 7 μM Congo red solution to a final concentration of 20 μM and incubated at room temperature for 30 min. Absorbance spectra were measured with a U-2810 spectrophotometer (Hitachi, Tokyo, Japan).

Results

Secondary structures of HN and D-Ser-containing HN

To investigate the structure of HN and its derivatives, the CD spectra of HN, [D-Ser^{7}]HN, [D-Ser^{14}]HN, [D-Ser^{7,14}]HN, and HNA in PBS were measured and used to calculate the secondary structure contents of the peptides (Table 1). For HN and D-Ser^{14}-containing HNs, the estimated content values for β-sheets and random coils were high. The β-sheet content of HN was 40.8 ± 1.0%. [D-Ser^{14}]HN and [D-Ser^{7,14}]HN showed a similar β-sheet content to that of HN in PBS. On the other hand, [D-Ser^{7}]HN and HNA showed significantly lower β-sheet contents ($P < 0.01$ and 0.001, respectively) and significantly higher random coil contents than HN ($P < 0.01$ and 0.001, respectively) (Table 1). Furthermore, there was no α-helical structure in [D-Ser^{7}]HN or HNA dissolved in PBS.

Next, to examine the structure of D-Ser-containing HN under lipophilic conditions, which mimic the environment of biological organs, the CD spectrum of each HN derivative was measured in 40% TFE/PBS. The spectra of all HN derivatives showed a strong negative peak at around 205 and 222 nm (Fig. 1), indicating an increase in the α-helical structures of HN, HNA, and all D-Ser-containing HNs tested under this condition. Under this condition, HN had an α-helix content of 10.8 ± 0.6%, which was similar to those of [D-Ser^{14}]HN and [D-Ser^{7,14}]HN (Table 1). On the other hand, [D-Ser^{7}]HN and HNA had higher α-helical contents than did HN. Furthermore, the β-sheet content of all HNs increased in 40% TFE/PBS compared with those in

Table 1 Contents of the secondary structure of humanin (HN) and its derivatives in PBS or 40% TFE/PBS

	HN	D-Ser^{7}	D-Ser^{14}	D-Ser^{7,14}	HNA
PBS					
α-Helix	2.3 ± 0.7	0.0 ± 0.0*	4.8 ± 0.4*	0.2 ± 0.1*	0.0 ± 0.0*
β-Sheet	40.8 ± 1.0	30.0 ± 2.6**	39.8 ± 1.0	43.5 ± 2.6	24.4 ± 0.5***
β-Turn	21.0 ± 1.4	22.7 ± 0.5	21.8 ± 0.7	17.3 ± 0.5*	20.7 ± 0.8
Random	35.9 ± 1.3	47.3 ± 2.4**	33.7 ± 0.4	39.0 ± 2.7	54.9 ± 1.2***
40% TFE/PBS					
α-Helix	10.8 ± 0.6	15.6 ± 2.7	13.5 ± 0.7*	11.0 ± 1.8	16.7 ± 2.2**
β-Sheet	44.4 ± 2.4	34.1 ± 6.4	42.6 ± 1.7	43.8 ± 4.8	28.1 ± 2.8**
β-Turn	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 2.6
Random	44.9 ± 1.9	50.3 ± 3.7	44.0 ± 1.1	45.2 ± 3.1	54.0 ± 1.5**

Data are shown as mean ± SEM ($n = 4$)
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with HN

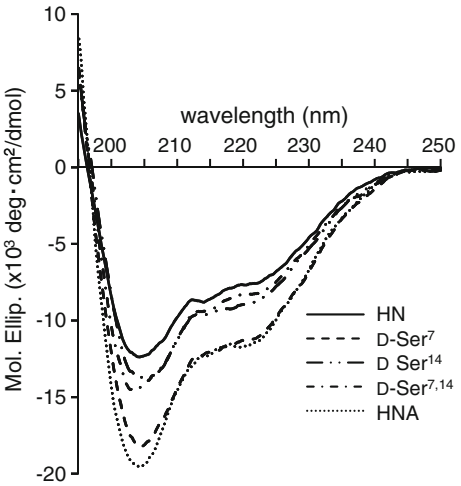

Fig. 1 CD spectra of HN, [D-Ser^{7}]HN, [D-Ser^{14}]HN, [D-Ser^{7,14}]HN, and HNA in 40% TFE/PBS. CD spectra were recorded at 0.1-nm intervals, 100 nm/min

PBS. These results suggested that HN and D-Ser-containing HN change their structures depending on the environmental conditions. In particular, [D-Ser^{7}]HN and HNA tend to change to a more flexible structure compared with those of other HN derivatives.

Fibril formation by HN derivatives

Our CD spectra data showed that HN and D-Ser-containing HN formed β-sheets in PBS and in 40% TFE/PBS. To examine whether HN and its derivatives formed fibrils based on this laminated β-sheet structure, we performed a Th-T assay. Th-T binds fibrils and increases its fluorescence intensity at a wavelength of around 480 nm. The fluorescence intensities of HN, [D-Ser^{7}]HN, and [D-Ser^{14}]HN in PBS were almost all the same (17.8 ± 8.7, 14.0 ± 5.6, and 15.0 ± 6.5, respectively) (Fig. 2a). In contrast, [D-Ser^{7,14}]HN exhibited higher fluorescence intensity than other HN peptides (58.6 ± 9.1, $P < 0.05$ compared with HN, $P < 0.01$ compared with [D-Ser^{7}]HN or [D-Ser^{14}]HN by one-way ANOVA) (Fig. 2a). In 40% TFE/PBS, [D-Ser^{7,14}]HN presented higher fluorescence intensity (70.6 ± 19.5) than other HN peptides again (Fig. 2b). All HN peptides exhibited higher fluorescence intensity under lipophilic condition compared with that in PBS (Fig. 2a, b). These results indicated that HN and D-Ser-containing HNs made fibrils and that, in the case of [D-Ser^{7,14}]HN, fibril formation was enhanced under both hydrophilic and lipophilic conditions.

We also performed a Congo red binding assay to examine whether the fibrils of HNs resembled amyloid

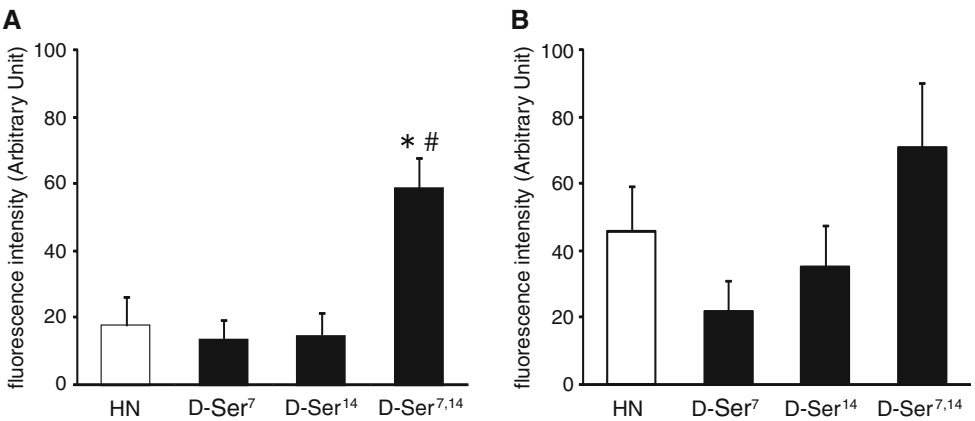

Fig. 2 Th-T assay of HN, [D-Ser^{7}]HN, [D-Ser^{14}]HN, and [D-Ser^{7,14}]HN in PBS (**a**) or 40% TFE/PBS (**b**). HN or HN derivatives are dissolved in PBS or 40% TFE/PBS to a concentration of 20 μM. After each HN peptide was incubated at 37°C for 2 h, the fluorescence intensity of the Th-T-bound HN peptide was measured. Data represent mean ± SEM for four experiments; *$P < 0.05$ compared with HN, $^{\#}P < 0.01$ compared with [D-Ser^{7}]HN or [D-Ser^{14}]HN

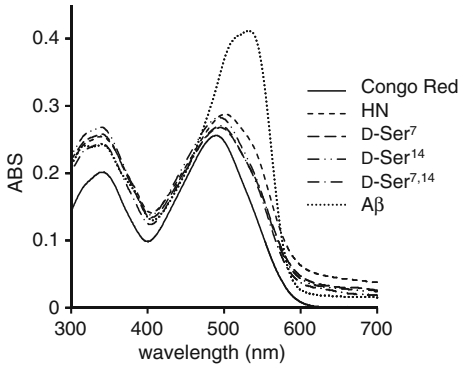

Fig. 3 Absorbance spectra of Congo red in the presence or absence of HN or each D-Ser-containing HN. HN or each HN derivative, dissolved in PBS, was diluted in 7 μM Congo red solution to a concentration of 20 μM. After each HN peptide was incubated at room temperature for 30 min, absorbance spectra (*ABS*) were measured. Aβ is shown as a positive control for the spectrum shift that accompanies amyloid fibril formation

fibrils. Congo red binds to amyloid-like fibrils that have β-sheets arranged in a regular parallel structure; this can be observed by a red shift of the Congo red absorbance spectrum (as shown in Fig. 3, Aβ). Analysis of the spectrum of Congo red in the presence of each HN derivative showed that the maximum absorbance shifted slightly from 490 nm to 494–496 nm (D-Ser-containing HN) and to 501 nm (HN), with a slight increase in absorbance (Fig. 3). These shifts were very slight compared with that of Aβ, indicating that only a small portion of HN and D-Ser-containing HNs formed amyloid-like fibrils in PBS and that HN and D-Ser-containing HNs formed fibrils that differed in shape from Aβ.

Discussion

We demonstrated here that HN and D-Ser-containing HNs form fibrils. We also found that HN, [D-Ser^{14}]HN, and [D-Ser^{7,14}]HN fold to form a stable structure with a high β-sheet content, whereas the structure of [D-Ser^{7}]HN and HNA changes flexibly according to the environmental conditions. Although proteins were once thought to comprise only L-amino acids, recent studies have revealed the existence of D-amino acid-containing proteins in vertebrates, especially in aged tissues (Fisher et al. 1986; Fujii et al. 1994). Because of their conformation, Asp and Ser residues isomerize more readily than other amino acid residues (Fujii et al. 1999). Therefore, we investigated the structural and physicochemical features of D-Ser-containing HN.

Amyloid fibrils such as Aβ and prion protein consist of the β-sheet structure. Our results show [D-Ser^{7,14}]HN had higher β-sheet content and formed more fibrils than did HN and other HN derivatives, indicating that fibril formation of HN peptides depends on the β-sheet content. Under lipophilic condition, the β-sheet content of each HN peptide slightly increased in comparison with that in PBS. Then, fibril formation of each HN peptide increased according to its change of the β-sheet content, respectively. The β-sheet contents of HN and D-Ser^{14}-containing HNs are high values both in PBS and in 40% TFE/PBS, showing that β-sheet structures of these HN peptides are stable under both hydrophilic and lipophilic conditions. This stable β-sheet structure is correlated with fibril formation. On the other hand, the α-helical structure of each HN peptide under lipophilic condition does not link to fibril formation.

In addition to the content of the β-sheet structure, the conformation of HN protein may be also related to fibril formation. Benaki et al. (2005) determined the three-dimensional structure of HN. HN holds two bends at around Ser^{7} and Ser^{14}, and the extended conformation between Ser^{7} and Ser^{14} is where the β-sheet structure is formed. Substitution of D-Ser for L-Ser implies a configurational inversion of the side-chain. This inversion may be accompanied by conformational changes around both bending regions. Therefore, this conformational change may facilitate interactions with [D-Ser^{7,14}]HN, resulting in the increased level of fibril formation by [D-Ser^{7,14}]HN. The biological meanings of fibrils of HN peptides, such as affinity with its receptors and its neuorprotective activity, need to be elucidated in the future.

HN, [D-Ser^{14}]HN, and [D-Ser^{7,14}]HN folded into β-sheets in PBS, and the α-helical content of the structure of these HN derivatives increased under lipophilic conditions. HN has neuroprotective activity against 25 μM Aβ1-43 with an IC_{50} of 100 nM–1 μM, whereas [D-Ser^{14}]HN and [D-Ser^{7,14}]HN are effective with an IC_{50} of 100 pM–1 nM, which is 1,000-fold higher than that of HN and the same as that of HNG (Terashita et al. 2003). HNG also formed a β-sheet structure in PBS, whereas the addition of TFE resulted in an increase in α-helical content (unpublished data), which is consistent with our findings with [D-Ser^{14}]HN and [D-Ser^{7,14}]HN. These data indicate that HN derivatives, including [D-Ser^{14}]HN, [D-Ser^{7,14}]HN, and HNG, which show potent neuroprotective activity, have similar structural characteristics. On the other hand, [D-Ser^{7}]HN and HNA were unstructured in PBS, whereas in 40% TFE/PBS their α-helical content increased dramatically. The structural flexibility of [D-Ser^{7}]HN tended to change according to the environmental conditions. HNA, which lacks neuroprotective activity, showed similar structural flexibility to that of [D-Ser^{7}]HN, indicating that these two peptides share the structural features that allow changes in flexibility in response to the environment.

In conclusion, we demonstrated the structural and physicochemical characteristics of HN, [D-Ser^{7}]HN,

[D-Ser^{14}]HN, and [D-Ser^{7,14}]HN. Isomerization of the Ser residues at positions 7 and 14 in HN induced a conformational change that led to an increase in fibril formation. Furthermore, HN and its derivatives could be classified into two classes according to their neuroprotective activity and physicochemical features. Our findings also show that the conformational characteristics of [D-Ser^{7,14}]HN may be important for its potent neuroprotective activity.

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