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Short communication

Prolyl endopeptidase inhibitory activity of chitosan sulfates with different degree of deacetylation

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Abstract

Three kinds of partially deacetylated chitosan, 90% deacetylated chitosan, 75% deacetylated chitosan and 50% deacetylated chitosan, were prepared from crab chitin by *N*-deacetylation with 40% (w/w) sodium hydroxide solution for different durations. In order to improve biological activity and solubility, their sulfated derivatives were prepared, and prolyl endopeptidase (PEP) inhibitory activities were investigated. Fifty percent-deacetylated chitosan sulfate (50-CS) exhibited the highest inhibitory activity, and inhibition rate was a dose-dependant. In addition, Dixon plots suggested that 50-CS was act as competitive inhibitor, and the inhibition constant (K_i) was 2.6 mg/ml.

Keywords: Hetero-chitosans; Sulfated derivatives; Prolyl endopeptidase; Competitive

1. Introduction

Prolyl endopeptidase (PEP, EC 3.4.21.26) is a proline-specific endopeptidase with a serine-type mechanism, hydrolyzes peptide bonds at the carboxyl terminus of prolyl residues, and was first found as an oxytocin-inactivating enzyme in the human uterus (Walter, Shlank, Glass, Schwartz, & Kerenyi, 1971). In the central nervous system, PEP has been proposed to play a role in the metabolism of proline-containing neuropeptides involving in the process of learning and memory such as thyrotropin releasing hormone (TRH), arginine-vasopressin (AVP), and substance P (SP) (Blumberg, Teichberg, Charli, Hersh, & McKelvy, 1980; Yoshimoto, Simmons, Kita, & Tsuru, 1981). In addition, the PEP activity of Alzheimer's patients was significantly higher than the normal (Aoyagi et al., 1990), and a putative amyloid A4-generating enzyme in Alzheimer's disease, was identified as PEP (Ishiura et al., 1990). Therefore, PEP inhibitors are expected to use therapeutic agents for progressive memory deficits and cognitive dysfunction related to aging and neurodegenerative diseases of the central nervous system.

Chitosan, which is a copolymer consisting of β-(1→4)-2-acetamido-D-glucose and β-(1→4)-2-amino-D-glucose units, is derived from chitin by deacetylation in the presence of alkali. It exhibits a wide variety of biological activities (Jeon & Kim, 2001; Maezaki et al., 1993; Park, Je, Byun, Moon, & Kim, 2004; Park, Je, & Kim, 2004; Suzuki et al., 1986). Owing to the biological functions, it is attracted much attention, however, the apptications of chitosan are limited because of the insolubility. Therefore, it is important to improve the solubility of chitosan. In recent years, there many water-soluble chitosan derivatives are reported (Lim & Hudson, 2004; Xie, Xu, & Liu, 2001). In addition, sulfated chitosan was prepared to develop anticoagulant activity (Drozd et al., 2001; Huang, Du, Yang, & Fan, 2003; Vongchan, Sajomsang, Kasinrerk, Subyen, & Kongtawelert, 2003). However, there is little information other biological activity.

In the present study, we prepared chitosan sulfates with different degree of deacetylation such as 90% deacetylated chitosan sulfate (90-CS), 75% deacetylated chitosan sulfate (75-CS), and 50% deacetylated chitosan sulfate (50-CS), and evaluated their PEP inhibitory activity.

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2. Material and methods

2.1. Materials

Chitin prepared from crab shells was donated by Kitto Life Co. (Seoul, Korea). PEP (Flavobacterium meningosepticum origin) and its substrate, benzyloxycarbonyl-glycyl-L-prolyl-*p*-nitroanilide (Z-Gly-Pro-*p*NA), were purchased from Seikagaku Co. (Tokyo, Japan). All other reagents were of the highest grade available commercially.

2.2. Preparation of hetero-chitosans

Three kinds of partially deacetylated chitosan, 90, 75, and 50% deacetylated chitosan, were prepared from crab chitin by *N*-deacetylation with 40% (w/v) sodium hydroxide solution according to our previous method (Park et al., 2004).

2.3. Preparation of sulfated hetero-chitosans

Sulfated chtiosans were prepared according to our previous method (Park, Je, Jung, Anh, & Kim, 2004). Briefly, chitosan (10 g) was dispersed in 1 l of distilled water, and treated with 2.2 g of sodium carbonate anhydrous and 4.5 g of trimethylamine-sulfur trioxide (Me_3N–SO_3). The mixture solution was heated at 65 °C for 12 h. The resultant solution was cooled then dialyzed exhaustively against distilled water using an electrodialyzer (Micro Acilyzer G3, Asahi Chemical Industry Co., Tokyo, Japan), and lyophilized.

2.4. Assay for PEP inhibitory acitivity

PEP activity was assayed using the methods of Yoshimoto, Walter, and Tsuru (1980) with minor modifications. A mixture of 10 μl of 0.1 M phosphate buffer (pH 7.0), 200 μl of sample, and 20 μl of 2 mM Z-Gly-Pro-*p*NA in 40% 1,4-dioxane was pre-incubated at 37 °C for 10 min. The reaction was started by adding 20 μl of 0.1 unit/ml PEP at 37 °C. After incubation for 30 min, the amount of released *p*-nitoaniline was determined colorimetically based on the absorbance at 410 nm using ELISA reader (A). A_{410} of the mixture containing 50 μl of buffer and 200 μl of sample was separately measured as above (B). A control was made by adding 200 μl of buffer instead of sample solution of (A). PEP inhibitory activity was calculated as follow: PEP inhibitory activity (%) = (A_{410} of Control − ($A - B$)/A_{410} of Control) × 100.

Table 1
The degree of deacetylation of chitosan prepared from crab chitin

Chitosans	Titration	IR
50% chitosan	51.7	47.6
75% chitosan	74.2	75.1
90% chitosan	90.7	88.3

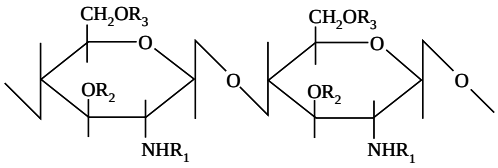

Fig. 1. Chemical structure of chitosan sulfate.

2.5. Statistics

The data presented are means ± SE of three determinations.

3. Results and discussion

Chitosans with different degree of deacetylation were prepared successfully according to our previous method, and degree of deacetylation was measured by titration method and IR spectroscopy (Park et al., 2004). Deacetylation ratio designated as 90, 75, and 50% (Table 1). In order to improve the solubility, sulfated chitosans were prepared according to our previous method (Park et al., 2004). The sulfated chitosan were obtained in over 90% yields as a white, fluffy, water-soluble material, and degree of substitution was 0.76. Characteristic absorptions derived from sulfo groups in the IR spectrum at 800, 1240, and 1350 cm^{-1} were assigned to C–O–S, S=O, and S–N, respectively (data not shown). Their chemical structure was shown in Fig. 1. The PEP inhibitory activity of chitosan sulfates (CS) with different degree of deacetylation is shown in Fig. 2. Three CS have obvious inhibitory activity, and 50-CS exhibited

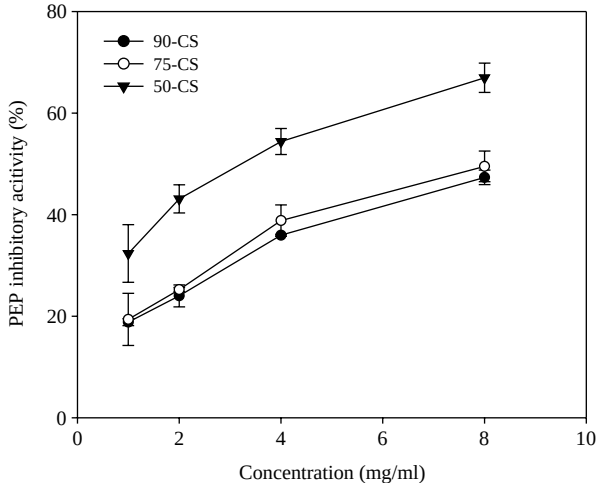

Fig. 2. PEP inhibitory activities of sulfated hetero-chitosans. 90-CS, 90% deacetylated chitosan sulfate; 75-CS, 75% deacetylated chitosan sulfate; 50-CS, 50% deacetylated chitosan sulfate. Values represent means ± SE ($n = 3$).

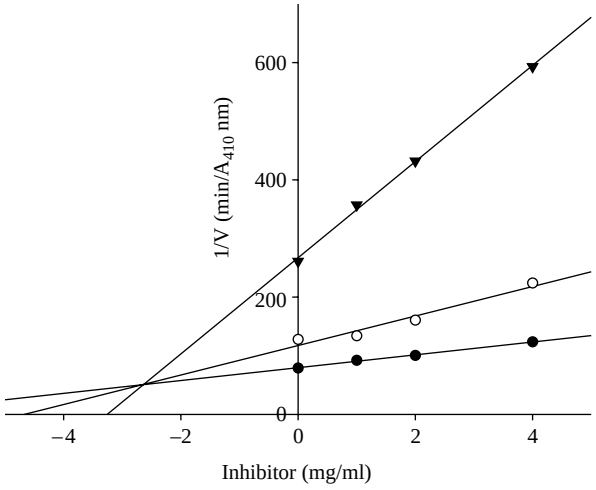

Fig. 3. Dixon plots of 50-CS. Substrate concentration: ▼, 1.0 mM; ○, 5.0 mM; ●, 10 mM.

the highest inhibitory activity. Their inhibition rate showed a dose dependant manner. In addition, the inhibition pattern of 50-CS was investigated using Dixon plots (Fig. 3). The results showed that 50-CS was competitive inhibition with a substrate, and the inhibition constant (K_i) of 50-CS was 2.6 mg/ml.

In recent years, many researchers have been developing chitosan derivatives to improve solubility and their biological activity. Xie et al. (2001) prepared water-soluble chitosan derivatives, and tested their antioxidant activity. They also reported antibacterial activity against *S. aureus* and *E. coli* (Xie, Xu, Wang, & Liu, 2002). Matsugo et al. (1998) reported the antioxidant activity of chitosan derivatives prepared by acylation. Especially, sulfated chitosan derivative were developed to improve their anticoagulant activity (Drozd et al., 2001; Huang et al., 2003; Vongchan et al., 2003). In our laboratory, sulfated hetero-chitosans were prepared with general method, and evaluated their anticoagulant activity (Park et al., 2004). However, other biological activities of sulfated chitosan have not been fully understood. Furthermore, biological activities of water-soluble chitosan derivatives with different deacetylation were not performed.

In this study, we investigated PEP inhibitory activities of sulfated hetero-chitosans with different deacetylation value, and 50-CS exhibited the highest inhibitory activity. In addition, Dixon plot suggested that 50-CS act as a competitive inhibitor. Most of the PEP inhibitors described in the literatures are structurally related to Z-prolyl-prolinal (Wilk & Orlowski, 1983) and the PEP inhibitory activity of sulfated hetero-chitosans is lower than those of other synthetic inhibitors. However, it is meaningful in that this is the first report on chitosan derivative as a PEP inhibitor. Moreover, these results might be useful for development of a new type of PEP inhibitor from chitosan derivatives for future work.

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