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Sulfated chitooligosaccharides as prolyl endopeptidase inhibitor

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Abstract

Prolyl endopeptidase (PEP, EC 3.4.21.26) is a proline-specific endopeptidase with a serine-type mechanism, which digests small peptide-like hormones, neuroactive peptides, and various cellular factors. PEP has been involved in neurodegenerative disorders, therefore, the discovery of PEP inhibitors can revert memory loss caused by amnesic compounds. In this study, we prepared hetero-chitooligosaccharides (COSs) with different molecular sizes using ultrafiltration (UF) membrane reactor system from hetero-chitosan with different degrees of deacetylation (DD; 90%, 75% and 50% deacetylation), and synthesized sulfated COSs (SCOSs). PEP inhibitory activities of SCOSs were evaluated and the results showed that 50% deacetylated SCOSs (50-SCOSs) exhibited higher inhibitory activities than those of 90% and 75% deacetylated SCOSs (90-SCOSs and 75-SCOSs). Among the 50-SCOSs (50-SCOS I, 5000–10,000 Da; 50-SCOS II, 1000–5000 Da; 50-SCOS III, below 1000 Da), 50-SCOS II possessed the highest inhibitory activity and IC_{50} value was 0.38 mg/ml. Kinetics studies with 50-SCOS II indicated a competitive enzyme inhibition with a K_i value of 0.78 mg/ml. It was concluded that the 50-SCOS II may be useful for PEP inhibitor and for developing a new type PEP inhibitor from carbohydrate based materials.

Keywords: Chitooligosaccharide; PEP; Sulfated; Kinetics

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. PEP was first found in human uterus as an oxytocin-cleaving enzyme [1], and was named post-proline cleaving enzyme (PPCE), since it cleaves peptides at the C-side of proline residue. It has been purified and characterized from various mammalian and bacterial sources. In human it is broadly distributed in all tissues with an especially high activity found in the brain [2]. However, the exact functions of PEP remain obscure in spite of its thorough enzymological and structural characterization. Therefore, many attempts have been focused on elucidation of the physiological role of the enzyme.

Several studies revealed that PEP involved in neurodegenerative disorders. 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) [3,4]. In addition, the PEP activity of Alzheimer's patients was significantly higher than the normal [5] and a putative amyloid A4-generating enzyme in Alzheimer's disease was identified as PEP [6]. 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.

Chitooligosaccharides (COSs) are derivative of chitosan and can be obtained by either chemical or enzymatic hydrolysis of chitosan. It has been received much attention as biomedical materials since it exhibited various biological activities, such as antitumor [7], immuno-stimulating [8], antifungal [9] and antimicrobial activity [10]. In recent years, a great number of

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derivatives were developed to improve their biological activities, specially sulfated chitosan and COSs derivatives were synthesized for the production of heparin-like blood anticoagulants [11–15]. Other biological activities, such as antimicrobial [16], anti HIV-1 [17] and heavy metal adsorbing activity [18] are also reported. As part of our ongoing investigation of biological activity from COSs derivatives, we have prepared COSs with different degree of deacetylation (DD) and molecular weight (MW), and synthesized sulfated COSs derivatives for the evaluation of their biological activity against PEP inhibitory capacity because of the increase biomedical applications of chitosan, COS and/or their derivatives. Therefore, the aim of this study was to investigate PEP inhibitory activity and was to evaluate factor affecting PEP inhibitory activity using different DD and MW of sulfated COS.

2. Experimental

2.1. Materials

Chitin prepared from crab shells was donated by Kitto Life Co. (Seoul, Korea). The chitosanase (35,000 U/g protein) derived from *Bacillus* sp. was purchased from Amicosen Co. (Jinju, Korea), and cellulase was donated by Pacific Chemical Co. (Seoul, Korea). An ultrafiltration (UF) membrane reactor system (Minitan^{TM}) for the production of hetero-chitooligosaccharides (hetero-COSs) was purchased from Millipore Co. (Bedford, MA, USA). 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 and their chitooligosaccharides (COSs)

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 and degree of deacetylation were determined by titration method and IR spectroscopy [19]. In addition, hetero-COSs, which are COSs prepared from 90%, 75%, and 50% deacetylated chitosans, were prepared by hydrolysis of hetero-chitosans in an UF membrane reactor system according to our previous method [20]. Briefly, a 1% solution of hetero-chitosans was prepared by dissolving 100 g hetero-chitosans in 1.0 l distilled water and 550 ml 1.0 M lactic acid, and the volume was made up to 10.0 l with distilled water. The pH was adjusted to be 5.5 with a saturated sodium carbonate solution. The UF membrane reactor system was used for fractionation of hetero-COSs. Ninety percent deacetylated chitosan was hydrolyzed with an endo-type chitosanase (35,000 U/g protein) with a substrate-to-enzyme ratio of 1:1.5 units for 36 h in a batch reactor, and was then heated at 98 °C for 10 min to inactivate the enzyme. Thereafter, the hydrolysates were separated using an UF membrane reactor system. The UF membranes used in the system were molecular weight cutoffs (MWCO) 10, 5, and 1 kDa, respectively. Seventy-five percent, and 50% deacetylated chitosans were hydrolyzed with a substrate-to-enzyme ratio of 1:5 units and of 1:10 units by cellulose (critical micelle concentration 100,000 U/g protein) for 2 h at 43 ± 2 °C in a batch reactor. Subsequently, chitosanase was added in the reactor, hydrolyzed for 36 h at the same temperature, and separated by an UF membrane reactor system. The hetero-COSs were fractionated into nine kinds of COSs with relatively high molecular weights (5000–10,000 Da; 90-COS I, 75-COS I, and 50-COS I), medium molecular weights (1000–5000 Da; 90-COS II, 75-COS II, and 50-COS II), and low molecular weights (below 1000 Da; 90-COS III, 75-COS III, and 50-COS III). All COSs recovered were lyophilized on a freeze-drier for 5 days.

2.3. Preparation of sulfated hetero-chitooligosaccharides

Sulfated chitooligosaccharides (SCOSs) were prepared according to our previous method [21]. Briefly, COSs (10 g) were 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 and 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 activity

PEP activity was assayed using the methods of Yoshimoto et al. [22] 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*-nitroaniline was determined colorimetrically 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. The IC_{50} value was defined as the concentration of sulfated chitooligosaccharides that is required to reduce PEP activity by 50%.

2.5. Kinetics

The type of inhibition was determined from Dixon plot [23], which is frequently used for both identification of the likely mechanism of enzyme inhibition and for estimation of K_i. Plots were prepared of the reciprocal of rate of metabolite formation ($1/v_{obs}$) versus inhibitor concentration at each substrate concentration. The point of intersection of these pairs of lines represents the value of K_i.