Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Polymers 60 (2005) 483–487
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Inhibitory activity of phosphorylated chitooligosaccharides on the formation of calcium phosphate

Se-Kwon Kim[a,*], Pyo-Jam Park[b], Won-Kyo Jung[a], Hee-Guk Byun[b], Eresha Mendis[a], Young-In Cho[c]

[a]Department of Chemistry, Pukyong National University, 599-1, Daeyon 3-dong, Nam-Gu, Busan 608-737, South Korea
[b]Department of Biotechnology, Kunkuk University, Chungju 308-701, South Korea
[c]Department of Food Science and Technology, Catholic University of Daegu, Hayang 712-702, South Korea

Received 27 October 2004; revised 26 February 2005; accepted 4 March 2005

Abstract

Chitooligosaccharides (COSs) were successfully prepared using an ultrafiltration membrane bioreactor system, and named as COS-I (10–5 kDa), COS-II (5–3 kDa), COS-III (3–1 kDa), and COS-IV (below 1 kDa), respectively. In addition, their phosphorylated derivatives were prepared by a P_2O_5 in methanesulfonic acid solution, and inhibitory activity on the formation of insoluble calcium phosphate of phosphorylated chitooligosaccharides. Phosphorylated COS-IV exhibited the highest inhibitory activity of calcium phosphate precipitation among tested chitooligosaccharides. Its inhibitory activity, especially at the concentration more than 4 mg/ml, was similar to that of CPP, which is widely used as a calcium fortifying agent increasing calcium absorbability. Therefore, phosphorylated chitooligosaccharides may be potential inhibitors of calcium phosphate precipitation.

Keywords: Calcium; Calcium phosphate; Inhibitor; Phosphorylated chitooligosaccharides

1. Introduction

Chitosan is a deacetylated polymer of *N*-acetylglucosamine, which is obtained after alkaline deacetylation of the chitin derived from the exoskeletons of crustaceans and arthropods. It has received considerable attention for its commercial applications in biomedical, food, and chemical industries. In addition, chitosan has been widely used in vastly diverse fields such as pharmaceutics, medicine and biotechnology (Muzzarelli, 1997). However, increasing attention has recently been given to convert its oligosaccharides because of their biological activities, such as antitumor activity (Jeon & Kim, 2002; Suzuki et al., 1986), immunostimulating effects (Jeon & Kim, 2001; Suzuki, Watanabe, Mikami, Matsumoto, & Suzuki, 1992), enhancing protective effects against infection with some pathogens in mice (Yamada, Shibuya, Kodama, & Akatsuka, 1993), antifungal activity (Kendra, Christian, & Hadwiger, 1989), antimicrobial activity (Hadwiger & Beckman, 1980; Hirano & Nagao, 1989; Jeon, Park, & Kim, 2001; Park, Je, Byun, Moon, & Kim, 2004; Park, Lee, & Kim, 2004), angiotensin I converting enzyme (ACE) inhibitory activity (Park, Je, & Kim, 2003a) and radical scavenging activity (Je, Park, & Kim, 2004; Park, Je, & Kim, 2003b, 2004).

Calcium, the most abundant mineral in the human body, has several important functions. More than 99% of total body calcium is stored in the bones and teeth where it functions to support their structure (Shils, 1999). The remaining 1% is found throughout the body in blood, muscle, and the fluid between cells. It is needed for muscle contraction, blood vessel contraction and expansion, the secretion of hormones and enzymes, and sending messages through the nervous system. Although, most people are aware that calcium is an important element in their bodies, calcium is severely deficient in most diets. When calcium intake is low or calcium is poorly absorbed, bone breakdown occurs because the body must use the calcium stored in bones to maintain normal biological functions such as nerve and muscle function. Its deficiency in the United

* Corresponding author. Tel.: +82 51 6206375; fax: +82 51 6288147.
E-mail address: sknkim@pknu.ac.kr (S.-K. Kim).

0144-8617/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2005.03.014

States has been shown as a major cause of osteoporosis, affecting approximately 26 million people annually (Melton, 1995). Therefore, the National Institute of Health (NIH) Consensus panel revised the recommendations for current calcium intake (NIH Consensus Development Panel, 1994). The optimal calcium intake has been estimated to be 800 mg/day during childhood below 5 years of age, 800–1000 mg/day for children from age 6 to 10, 1200–1500 mg/day for adolescence or young adults from age 12–24 and pregnant or lactating women, 1000 mg/day from age 25 to the time of estrogen deprivation or age 65, and 1500 mg/day for elderly people. In general, the basic source of calcium is the diet, and the most common and trusted source of calcium is milk or other dairy products (Anderson & Garner, 1996). However, many elderly Korean people do not drink milk due to lactose indigestion and intolerance which make them allergic to milk. As an alternative, these people prefer to drink calcium-fortified fruit juice, or to take calcium-rich foods and calcium supplements. Several studies have been performed in the last two decades on casein phosphopeptides (CPP), which may function as carriers for different minerals, especially calcium. It has been proposed that CPP, which form soluble complexes with $Ca_3(PO_4)_2$ in vitro, may lead to enhanced calcium absorption by limiting the precipitation of calcium at alkaline conditions pertaining in the distal ileum. Therefore, it is important to prevent the formation of calcium precipitate, to enhance calcium absorption.

In this study, the inhibitory activity on the formation of insoluble calcium phosphate of phosphorylated chitooligosaccharides prepared from chitosan oligosaccharides with different molecular weight was investigated.

2. Materials and methods

2.1. Materials

Chitin prepared from crab shells was donated by Kitto Life Co. (Seoul, South Korea). The chitosanase (35,000 U/g protein) derived from *Bacillus* sp. was purchased from Amicosen Co. (Jinju, Korea), and an ultrafiltration (UF) membrane reactor system (Minitan™) for production of hetero-COSs was from Millipore Co. (Bedford, MA, USA). All other reagents were the highest grade commercially available.

2.2. Preparation of chitooligosaccharides using an UF membrane bioreactor

Four kinds of chitooligosaccharides were prepared according to our previous method (Park et al., 2003a,b). One percent (1%) solution was prepared by dispersing 100 g of chitosan in 1.0 l of distilled water while stirring with 550 ml of 1.0 M lactic acid and making the final volume up to 10 l with distilled water. The pH of the solution was adjusted to 5.5 using saturated sodium hydrogen carbonate solution. Ninety-three (93%) deacetylated chitosan was hydrolyzed with an endo type chitosanase (35,000 U/g protein) with substrate to enzyme ratio of 1.0–1.5 for 18 h in a batch reactor, and then heated at 98 °C for 10 min to inactivate the enzyme. Thereafter, hydrolysates were separated using an UF membrane reactor system. UF membranes used in the system had molecular weight cut off (MWCO) 10, 5, 3, and 1 kDa, respectively. The chitooligosaccharides recovered were lyophilized in a freeze-dryer (model PVTFD50A, Ilsin Lab. Co., Korea) for 5 days.

2.3. Preparation of phosphorylated chitooligosaccharides

Chitooligosaccharides were phosphorylation by P_2O_5 in methanesulfonic acid solution according to the procedure mentioned by Nishi et al. (1986). The reaction mixture was filtrated and the filtrate was dialyzed thoroughly against distilled water to remove small molecular substances. After concentration by vacuum evaporation and lyophilization, solid phosphorylated chitooligosaccharides was obtained.

2.4. Evaluation on the inhibition of calcium phosphate formation by measuring the amount of calcium phosphate precipitated

The assay was performed according to the methods of Hay, Moreno, and Schlesinger (1979) with slight modification. Twenty millimolar phosphate buffer (pH 7.4) was used as the phosphate source. A half-milliliter phosphate solution, 0.2 ml of 200 mM KCl to control the ionic strength, and 0.2 ml of 15 mM NaN_3 as an antiseptic were mixed in 1.5 ml polypropylene tubes. Chitooligosaccharide (0.2 ml, 2 mg/ml) was added to the solution at specified concentrations. Afterward, 0.25 ml of 20 mM $CaCl_2$ was added to the solution, immediately, it was incubated in shaker at 37 °C for 0–12 h. Each solution was then centrifuged at 8000*g* for 10 min, and the supernatant was diluted with a 0.1 N nitrate solution and stored. The calcium concentration in the diluted supernatant solution was measured by an inductively coupled plasma mass spectrometer (ICP-MS, Elan 6100, Perkin Elmer/SCIEX, Norwalk, USA).

2.5. Evaluation on the inhibition of calcium phosphate formation by sample concentration

Sample solution (0.2 ml; 8 mg/ml), 1.0 ml of 10 mM phosphate buffer (pH 7.4), and 0.25 ml of 4 mM $CaCl_2$ were mixed in tubes. The solution was incubated in a shaker at 37 °C for 3 h. Each solution was then centrifuged at 8000*g* for 10 min, and the supernatant was diluted with a 0.1 N nitrate solution and stored. The calcium concentration in the diluted supernatant solution was measured by ICP-MS. The percentage rate of Ca solubilization (%) was calculated as follows:

The percentage rate of Ca solubilization (%) = the amount of residual calcium in the supernatant solution/the amount of total calcium treated in the tube.

3. Results and discussion

The chitooligosaccharides were successfully prepared using an UF membrane bioreactor system with four different membranes, and the molecular weight profiles of each oligosaccharide showed a distinct decrease of molecular weights according to the pore size of membranes used with molecular weight cut-off (MWCO) 10.0, 5.0, 3.0, and 1.0 kDa, respectively. The chitooligosaccharides were named as COS-I, which is the chitooligosaccharides passed out through MWCO 10.0 kDa membrane, but not through 5.0 kDa, COS-II, which is the chitooligosaccharides passed out through MWCO 5.0 kDa membrane, but not through 3.0 kDa, COS-III, which is the chitooligosaccharides passed out through MWCO 3.0 kDa membrane, but not through 1.0 kDa, and COS-V, which is the chitooligosaccharides passed out through 1.0 kDa membrane. In general, the phosphorylation of many materials was performed by P_2O_5 in methanesulfonic acid solution (Nishi et al., 1986). In this study, chitooligosaccharide phosphates were obtained in over 90% yields as white and water-soluble materials. As shown in Fig. 1, the infrared spectra of the phosphorylated COS-I (P-COS-I) showed that peaks at 1650 and 1544 cm^{-1} from amide absorptions were still remaining. Hydroxyl group absorption in chitosans at 1318 cm^{-1} became weak while new P=O and P–O absorptions of phosphate group at 1258 and 1000 cm^{-1}, respectively, appeared gradually as the degree of substitution increased in phosphorylated samples. The hydroxyl groups at C-3 and

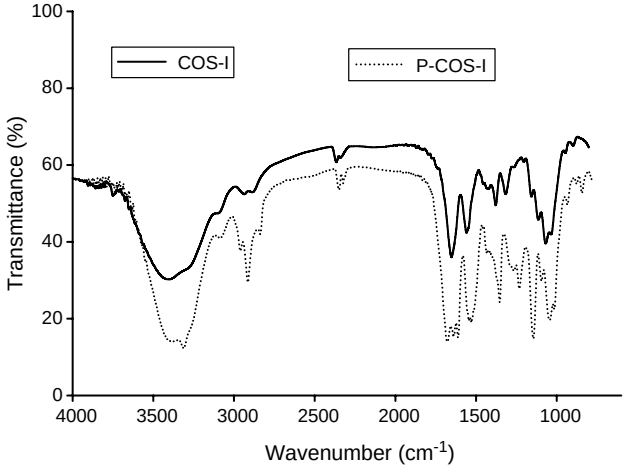

Fig. 1. The IR spectra of the chitooligosaccharide (COS) and phosphorylated chitooligosaccharide (COS-I). Peaks at 1650 and 1544 cm^{-1} illustrated by specific absorption and vibration of amide groups. Hydroxyl groups (–OH) and phosphate groups (P=O and P–O) in P-COS molecules led to arise peaks at 1318, 1258 and 1000 cm^{-1}.

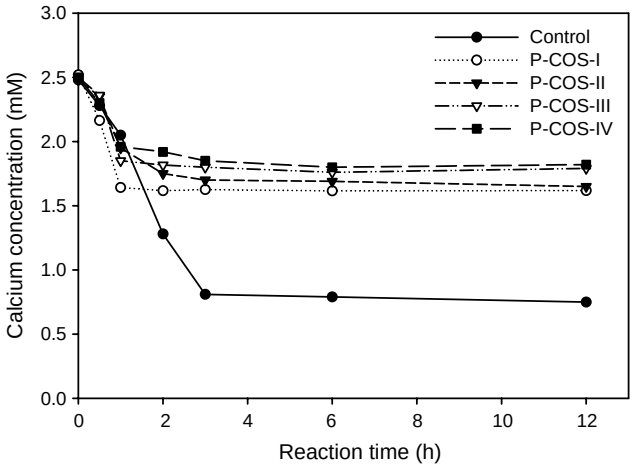

Fig. 2. Inhibitory of phosphorylated chitooligosaccharides against the formation of calcium phosphate.

C-6 of chitin polymer were mainly substituted with the phosphate group, and 10.6% (w/w) phosphorus was measured in the derivatives by elemental analysis as reported by Nishi et al. (1986). Wang, Ma, Wang, and He (2001) have suggested that some hydroxyl groups in chitosan were phosphorylated. All phosphorylated chitooligosaccharides exhibited the inhibitory activity of calcium phosphate formation (Fig. 2). The calcium concentration in the supernatant was suddenly decreased until 1 h, and became almost constant after 3 h. As the incubation time increased, the calcium concentration in the supernatant decreased. This result indicates that chitooligosaccharides delayed rather than prevented the calcium phosphate formation as reported by Hay et al. (1979), Termine and Posner (1970), and Yamamoto, Kumagai, Sakiyama, and Song (1992). Naito (1986) reported that the calcium concentration in the supernatant was linear with the logarithm of poly-L-glutamate concentration, and Williams and Sallis (1979) reported that citrate was a poor inhibitor over the citrate concentration range tested (10–200 μM). In addition, Yamoto et al. (1992) reported that alginates exhibited an inhibitory activity against calcium phosphate formation. In addition, several researchers reported on the effects of molecular weight against the inhibitory activity of calcium phosphate formation. Amjad (1990) reported that polydisperse polyacrylic acids has the maximal molecular weight (about 2000 Da) for inhibiting calcium phosphate insolubilization. Okamoto (1986) also stated that the chelating ability of PAA for calcium ions increased and the gelation of PAA by calcium ions was facilitated as the molecular weight was increased. In the present study, the inhibitory activity of calcium phosphate formation in the supernatant was not dependant on their molecular weights, and the maximal molecular weight was not found for the inhibitory activity against calcium phosphate

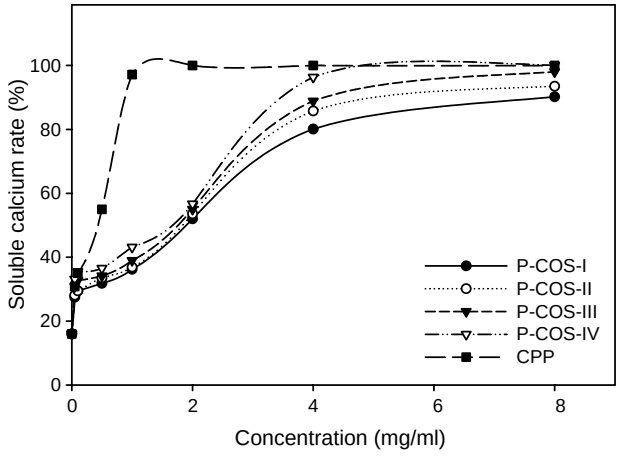

Fig. 3. Inhibitory effect of the formation of calcium phosphate on various concentrations of phosphorylated chitooligosaccharides. The percentage rate of Ca solubilization or soluble Ca (%) = the amount of residual calcium in the supernatant solution/the amount of total calcium treated in the tube.

insolubilization. Since phosphorylated chitooligosaccharides have no gelation tendency, the maxima might have disappeared. Fig. 3 shows the dependence of the calcium concentration in the supernatant on the various concentrations of chitooligosaccharides. In addition, P-COS-IV exhibited the highest inhibitory activity of calcium phosphate precipitation among tested chitooligosaccharides. Its inhibitory activity, especially at the concentration more than 4 mg/ml, was similar to that of casein phosphopeptide (CPP), which is widely used as a calcium agent. Many inhibitors of the formation of insoluble calcium phosphate have been reported, such as casein phosphopeptide, alginate, glutamate, and so on. However, there has been reported little information on the inhibitory activity of the phosphorylated chitosan polymers or oligomers against calcium phosphate formation until now. In addition, we have previously reported that we could not observe any toxic effects of the chitooligosaccharides in three groups of Sprague–Dawley rats given orally 500, 1000, and 2000 mg/kg per day weight change, general symptoms, food consumption, urinalysis, hematology, blood biochemistry, relative organ weights, and histopathological findings. In vivo study on toxicity of the chitosan derivative, P-COS, will be further performed to identify its bioavailability, and it may be potential inhibitors of calcium phosphate precipitation.

Acknowledgements

This research was funded by a grant (p-2004-02) from Marine Bioprocess Research of the Marine Bio 21 Center funded by the Ministry of Maritime Affairs & Fisheries, Republic of Korea.

References

Amjad, Z. (1990). Influence of polyelectrolytes on the precipitation of amorphous calcium phosphate. *Colloids Surfaces*, *48*, 95–106.

Anderson, J. J. B., & Garner, S. C. (1996). Calcium and phosphorous nutrition in health and disease. Introduction. In J. J. B. Anderson, & S. C. Garner (Eds.), *Calcium and phosphorous in health and disease* (pp. 1–5). New York: CRC Press.

Hadwiger, L. A., & Beckman, J. M. (1980). Chitosan as a component of pea–*Fusarium solani* interactions. *Plant Physiology*, *66*, 205–211.

Hay, D. I., Moreno, E. C., & Schlesinger, D. H. (1979). Phosphoprotein inhibitors of calcium phosphate precipitation from salivary secretions. *Inorganic Perspect Biology and Medicine*, *2*, 271–285.

Hirano, S., & Nagao, N. (1989). Effects of chitosan, pectic acid, lysozyme, and chitinase on the growth of several phytopathogens. *Agrical Biology and Chemistry*, *53*, 3065–3066.

Je, J. Y., Park, P. J., & Kim, S. K. (2004). Free radical scavenging properties of hetero-chitooligosaccharides using an ESR spectroscopy. *Food and Chemical Toxicology*, *42*, 381–387.

Jeon, Y. J., & Kim, S. K. (2001). Potential immuno-stimulating effect of antitumoral fraction of chitosan oligosaccharides. *Journal of Chitin and Chitosan*, *6*, 163–167.

Jeon, Y. J., & Kim, S. K. (2002). Antitumor activity of chitosan oligosaccharides produced in ultrafiltration membrane reactor system. *Journal of Microbiology and Biotechnology*, *12*, 503–507.

Jeon, Y. J., Park, P. J., & Kim, S. K. (2001). Antimicrobial effect of chitoligosaccharides produced by bioreactor. *Carbohydrate Polymers*, *44*, 71–76.

Kendra, D. F., Christian, D., & Hadwiger, L. A. (1989). Chitosan oligomers from *Fusarium solani*/pea interactions, chitinase/(β-glucanase digestion of sporelings and from fungal wall chitin actively inhibit fungal growth and enhance disease resistance. *Physiological and Molecular Plant Pathology*, *35*, 215–230.

Melton, L. J. (1995). How many women have osteoporosis now? *Journal of Bone and Mineral Research*, *10*, 175–177.

Muzzarelli, R. A. A. (1997). *Chitin*. Oxford: Pergamon Press.

Naito, H. (1986). The mechanism of enhancement in intestinal calcium absorption with phosphopeptides derived during case in digestion. *Journal of Japanese Society for Nutrition and Food Science 39*, 433–439.

NIH Consensus Conference (1994). NIH Consensus Development Panel on Optimal Calcium Intake. *The Journal of the American Medical Association 272*(24), 1942–1948.

Nishi, N., Ebina, A., Nishimura, S. I., Tsutsumi, A., Hasegawa, O., & Tokura, S. (1986). Highly phosphorylated derivatives of chitin, partially deacetylated chitin and chitosan as new functional polymers: Preparation and characterization. *International Journal of Biological Macromolecules*, *8*, 311–317.

Okamoto, M. (1986). Chelating ability and easy gelation of polyelectrolytes. *Nippon Kagaku Kaishi 9*, 1153–1160.

Park, P. J., Je, J. Y., Byun, H. G., Moon, S. H., & Kim, S. K. (2004). Antimicrobial activity of hetero-chitosans and their oligosaccharides with different molecular weights. *Journal of Microbiology and Biotechnology*, *14*, 317–323.

Park, P. J., Je, J. Y., & Kim, S. K. (2003a). Angiotensin I converting enzyme (ACE) inhibitory activity of hetero-chitooligosaccharides prepared from partially different deacetylated chitosans. *Journal of Agricultural and Food Chemistry*, *51*, 4930–4934.

Park, P. J., Je, J. Y., & Kim, S. K. (2003b). Free radical scavenging activity of chitooligosaccharides by electron spin resonance spectrometry. *Journal of Agricultural and Food Chemistry*, *51*, 4624–4627.

Park, P. J., Je, J. Y., & Kim, S. K. (2004). Free radical scavenging activities of differently deacetylated chitosans using an ESR spectrometer. *Carbohydrate Polymers*, *55*, 17–22.

Park, P. J., Lee, H. K., & Kim, S. K. (2004). Preparation of heterochitooligosaccharides and their antimicrobial activity on *Vibrio parahaemolyticus*. *Journal of Microbiology and Biotechnology*, *14*, 41–47.

Shils, M. E. (1999). *Modern nutrition in health and disease* (9th ed.). Baltimore, MD: Williams & Wilkins.

Suzuki, K., Mikami, T., Okawa, Y., Tokorom, A., Suzuki, S., & Suzuki, M. (1986). Antitumor effect of hexa-*N*-acetylchitohexaose and chitohexaose. *Carbohydrate Research*, *151*, 403–408.

Suzuki, S., Watanabe, T., Mikami, T., Matsumoto, T., & Suzuki, M. (1992). Immuno-enhancing effects of *N*-acetylchitohexaose. In C. J. Brine, P. A. Sanford, & J. P. Zikakis (Eds.), *Advances in chitin and chitosan* (pp. 277–316). Barking: Elsevier.

Termine, J. D., & Posner, A. S. (1970). Calcium phosphate formation in vitro. I. Factors affecting initial phase separation. *Archives of Biochemistry and Biophysics*, *140*, 307–317.

Wang, X., Ma, J., Wang, Y., & He, B. (2001). Structural characterization of phosphorylated chitosan and their applications as effective additives of calcium phosphate cements. *Biomaterials*, *22*, 2247–2255.

Williams, G., & Sallis, J. D. (1979). Structure–activity relationship of inhibitors of hydroxyapatite formation. *Biochemical Journal*, *184*, 181–184.

Yamada, A., Shibuya, N., Kodama, O., & Akatsuka, T. (1993). Induction of phytoalexin formation in suspension-cultured rice cells by *N*-acetylchitooligosaccharides. *Bioscience, Biotechnology and Biochemistry*, *57*, 405–409.

Yamoto, K., Kumagai, H., Sakiyama, T., & Song, C. M. (1992). Inhibitory activity of alginates against the formation of calcium phosphate. *Bioscience, Biotechnology and Biochemistry*, *56*, 90–93.