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Antioxidative Stress Activity of Oligophosphopeptides Derived from Hen Egg Yolk Phosvitin in Caco-2 Cells

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The protective effects of hen egg yolk phosvitin phosphopeptides (PPPs) against hydrogen peroxide (H_2O_2)-induced oxidative stress were evaluated in an in vitro assay using human intestinal epithelial cells. Caco-2 cells were stimulated with 1 mM H_2O_2 for 6 h, and the secretion of IL-8, a proinflammatory mediator, was determined by ELISA as a biomarker of oxidative stress. The inhibition of H_2O_2-induced IL-8 secretion from Caco-2 cells was observed by pretreatment for 2 h with PPPs, but not with phosvitin. PPPs also suppressed the formation of malondialdehyde in H_2O_2-treated Caco-2 cells. Furthermore, intracellular glutathione levels and glutathione reductase activity were elevated by the addition of PPPs. The protective effects of PPPs against H_2O_2-induced oxidative stress were almost the same as that of glutathione, and PPPs with a high content of phosphorus exhibited higher protective activity than PPPs without phosphorus; however, phosphoserine itself did not show any significant antioxidative stress activity. These findings suggest that oligophosphopeptides from hen egg yolk phosvitin possess novel antioxidative activity against oxidative stress in intestinal epithelial cells and that phosphorus and peptide structure seem to have a key role in the activity.

KEYWORDS: Phosvitin; phosphopeptides; egg yolk; oxidative stress; IL-8; Caco-2

INTRODUCTION

Oxidative stress is a state characterized by an excess of reactive oxygen species (ROS) in the body, which creates a potentially unstable cellular environment that is associated with tissue damage, accelerated aging, and degenerative diseases. ROS are highly reactive, and their accumulation induces cell damage by modifying molecules, including proteins, lipids, and DNA (*1*, *2*). Biological systems contain a group of naturally occurring compounds (antioxidants) to neutralize free radicals such as ROS and prevent cell damage; however, oxidative stress leads to an imbalance between antioxidants and oxidants (*3*). Many environmental factors are implicated in the generation of ROS, such as tobacco smoke, UV irradiation, overexercise, and the consumption of alcohol and certain foods (*4*).

There have been many reports on the antioxidant activity of dietary components. Catechin in green tea (*5*), lycopene in tomato (*6*), and polyphenols in red wine (*7*) are well-known natural antioxidants that behave as ROS scavengers, metal chelators, and enzyme modulators, and recent studies have demonstrated the protective effects of such compounds against oxidative stress using cell culture models. Echeverry et al. (*8*) reported that phenolic compounds in red wine exhibit cytoprotective activity against cell damage induced by oxidative stress. Manna et al. (*9*, *10*) showed that olive oil polyphenols can exert protective effects against hydrogen peroxide (H_2O_2)-induced oxidative cell injury in intestinal epithelia.

Gastrointestinal epithelial cells are exposed to oxidative stress that results in inflammation of the gut mucosa through the production of proinflammatory cytokines such as IL-8 and TNF-α (*11*−*13*). Especially, IL-8 production activates inflammatory cells (neutrophils), which extend the tissue damage (*14*, *15*). Thus, a reduction of IL-8 production may provide antioxidative protection against stress activity in gastrointestinal epithelial cells.

Hen egg yolk phosvitin is a highly phosphorylated protein with a molecular weight (MW) of 35000 that comprises 10% phosphorus and 6.5% carbohydrates (*16*). It contains 123 serine (Ser) residues that account for 57.5% of the total amino acid residues, and most of these Ser are monoesterified with phosphate (*17*). In our previous work, oligophosphopeptides (PPPs) with MWs of 1000−3000 were prepared from egg yolk phosvitin by partial alkaline dephosphorylation and tryptic hydrolysis. PPPs with 35% phosphate retention enhanced calcium- and iron-binding ability and inhibited the formation of phosphate precipitate (*18*−*20*). These findings suggest that PPPs represent a valuable new nutraceutical by increasing calcium and iron uptake in the intestinal tract. In contrast, it has been reported that casein phosphopeptides—phosphorylated fragments derived from the enzymatic hydrolysis of casein—not only enhance the bioavailability of calcium (*21*−*23*) but also show free radical scavenging activity and antioxidant activity against lipid peroxidation in ground beef homogenates

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(*24*, *25*). Thus, investigating the antioxidant activity of PPPs may aid the development from egg yolk phosvitin of a new and powerful antioxidant against oxidative stress on intestinal epithelia.

This study evaluated the protective effects of PPPs derived from hen egg yolk against H_2O_2-induced oxidative stress (as quantified by IL-8 secretion) in an in vitro assay using human intestinal epithelial cells (Caco-2 cells). We also measured glutathione (GSH) content and related enzyme activity to determine whether PPPs have any modulatory effects on the intracellular reduction−oxidation (redox) balance.

MATERIALS AND METHODS

Materials. Trypsin (EC 3.4.21.4; 11000 units/mg), hydrogen peroxide, butylated hydroxytoluene (BHT), GSH reductase (EC 1.6.4.2; GR), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Gibco Invitrogen (Burlington, ON, Canada). The flasks for growing cells were obtained from Corning Costar (Cambridge, MA).

Preparation of Phosvitin and Its Derivatives. Phosvitin was prepared from hen egg yolk according to the method of Losso and Nakai (*26*). Partially dephosphorylated phosvitin and its oligophosphopeptides were prepared according to the method of Jiang and Mine (*18*) with the following modifications. Phosvitin (1 g) was dissolved in 40 mL of 0.1 N NaOH and incubated at 37 °C for 3 h, and the suspensions were adjusted to a pH of 7.0. After overnight dialysis against Milli-Q water, the partially dephosphorylated phosvitin samples were lyophilized. PPPs were prepared by tryptic digestion. Partially dephosphorylated phosvitin (1 g) was suspended in 30 mL of Milli-Q water, and the pH of the solution was adjusted to 8.0 with 0.1 N NaOH. Trypsin was added to sample solutions at an enzyme-to-substrate ratio of 1:50 and then incubated overnight at 37 °C. The enzymatic reaction was stopped by heating at 90 °C for 10 min. The tryptic digests were centrifuged at 5900*g* for 15 min at 10 °C, and the supernatant was microfiltered through a 0.45-μm membrane and then ultrafiltered on a 1-kDa cutoff Diaflo membrane (Amicon, Beverly, MA) with Milli-Q water three times. The resulting retentate was lyophilized.

PPPs with different phosphorus content were separated using anion-exchange high-performance liquid chromatography (HPLC) with a Waters 1525 binary HPLC pump in combination with a Water in-line Degass AF, a Waters 717 Plus autosampler, and a Waters 2487 dual absorbance detector with Breeze software control. PPPs were injected into a Mono Q HR 5/5 anion exchange column (Pharmacia Biotech, Uppsala, Sweden) and eluted with 20 mM ammonium bicarbonate (pH 8.0) with a linear NaCl gradient from 0 to 1.0 M, at a flow rate of 1.0 mL/min. Effluents were monitored at 280 nm. Three fractions were collected and named PPP-1, PPP-2, and PPP-3. Their phosphorus contents were determined according to the method of Bartlett (*27*), with untreated phosvitin used as a control. Amino acid analysis was carried out using test samples hydrolyzed in 6 N HCl with 1% phenol at 110 °C for 24 h with a Waters Pico-Tag amino acid analyzer (Waters Millipore, Milford, MA).

Cell Culture. Caco-2 cell lines were used as a model of the intestinal epithelium, and this cell line was purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured in DMEM with 20% FCS in the presence of penicillin−streptomycin (50 units/mL) (Gibco Invitrogen), and incubated at 37 °C in 5% CO_2. Passages 20−50 were used in all monolayers employed in tissue oxidative-stress experiments. The medium was changed twice per week. The cells were grown in a flask for 5−8 days after seeding and then transferred (at 2×10^5 cells/mL) to 24-well culture plates (Corning Costar). The cells were cultivated for 5−7 days until confluent monolayers formed.

Induction of Oxidative Stress. The tissue oxidative stress was induced in confluent cell cultures by the addition of H_2O_2. Caco-2 monolayers were washed twice with Hank's balanced salt solution without calcium and magnesium and placed in 5% serum-containing media. The cells were then incubated for 2 h with various concentrations of PPP and its derivates (0−0.5 mg/mL) or GSH (0−1.0 mM) followed by the addition of 1 mM H_2O_2 for 6 h. At least 95% of the cells remained viable for the above treatments, as assessed by trypan-blue exclusion. The culture supernatants were collected to measure IL-8 production and stored at −80 °C. The cells were then washed twice with 100 mM phosphate (pH 7.2) containing 100 mM NaCl (phosphate-buffered saline, PBS) and suspended in PBS with 5 mM BHT (for determination of malondialdehyde, MDA), 0.1% Triton X-100 (for GSH measurement), and 100 mM PBS (pH 7.5) with 1 mM EDTA (for GR assay). Suspended cells were lysed on ice by sonication (Sonifier 250, Branson, Danbury, CT) and centrifuged at 10000*g* and 4 °C for 15 min. The supernatant was stored at −80 °C until use. The protein concentration in the cell lysate was determined according to the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin (BSA) as the standard.

IL-8 Immunoassay. Sandwich ELISA was performed to determine the levels of IL-8 produced in the culture supernatants. Briefly, 96-well ELISA microtiter plates (Corning Costar) were coated at 100 μL/well with mouse antihuman IL-8 antibody (BD Bioscience, San Diego, CA) (1:1000 dilution) in 100 mM sodium phosphate (pH 9.0) overnight at 4 °C and then washed three times with PBS containing 0.05% Tween-20 (PBST). Nonspecific binding sites were blocked by incubation with 2% BSA in PBS for 1 h at 37 °C. After three washings with PBST, culture supernatant samples were added to the wells (at 100 μL/well) and incubated for 2 h at 37 °C. After four washings, biotinylated mouse antihuman IL-8 antibody (BD Bioscience) (1:2000 dilution) was added to each well and incubated for 1 h at 37 °C. The plates were further washed four times with PBST and incubated with avidin−horseradish peroxidase conjugate (BD Bioscience) (1:3000 dilution) for 1 h at 37 °C. The plates were washed six times and developed with 100 μL/well of 3,3′,5,5′-tetramethylbenzidine (TMB) for 15 min at 37 °C. The reaction was terminated with 0.5 N H_2SO_4 (50 μL/well), and the absorbance at 450 nm was read by a microplate reader (model 550, Bio-Rad Laboratories).

Determination of MDA. The cellular content of MDA was measured to determine the extent of lipid peroxidation according to the method of Janero (*28*) with a modification. The cell lysate (0.2 mL) was mixed with 0.65 mL of 10 mM *N*-methyl-2-phenylindole and 0.1 mM $FeCl_2$ in acetonitrile. A total of 0.15 mL of 12 N HCl was added to the solution, which was then reacted for 1 h at 45 °C. After the solution had cooled to room temperature, the absorbance at 586 nm was measured using a spectrophotometer (UV-1201, Shimadzu, Kyoto, Japan). The MDA concentration was calculated using a standard curve with methanesulfonic acid and converted to moles of MDA per milligram of protein.

Measurement of Intracellular GSH. Intracellular total GSH was determined according to the method of Allen et al. (*29*) with a modification. The cell lysate was added to a 10% sulfosalicylic acid solution to precipitate proteins and centrifuged at 11000*g* for 10 min at 4 °C. The supernatant (25 μL) was mixed with 125 μL of 100 mM PBS containing 4 mM EDTA, 0.2 mM NADPH, and 0.5 mM DTNB and 100 units/mL GR. The mixture was incubated for 5 min at 25 °C, and the absorbance was measured at 412 nm. The concentration of GSH in the cell lysate was calculated using a standard curve and expressed as moles of GSH per milligram of protein.

Determination of GR Activity. GR activity was determined according to the procedure of Carlberg and Mannervik (*30*), by following the decomposition of NADPH at 340 nm and 25 °C. Briefly, 0.1 mL of cell lysate was mixed with 0.85 mL of 100 mM PBS (pH 7.5) containing 1 mM EDTA and 0.02 mM oxidized GSH (GSSG). A total of 0.05 mL of 0.1 mM NADPH was added to the solution, and the decrease in absorbance was recorded every minute for 10 min using a spectrophotometer. Changes in the rate of absorbance were converted into units of GR per milligram of protein using a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}. One unit of activity was defined as the oxidization of 1 μmol of NADPH per minute.

Statistical Analysis. Values are given as means ± SD of triplicate measurements. Statistical significance of the data was determined by Student's *t* test.

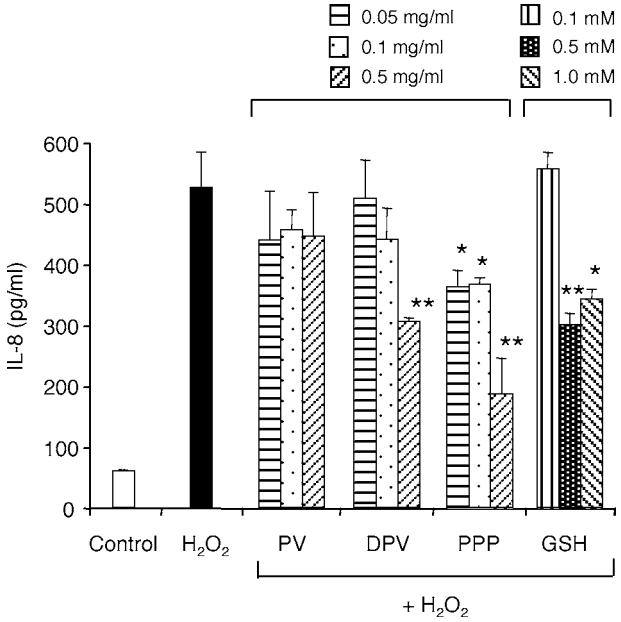

Figure 1. Effects of phosvitin and its derivatives on IL-8 secretion in H_2O_2-treated Caco-2 cells. Cells were cultured with 5% FBS–DMEM, treated with various concentrations of phosvitin and its derivatives (0–0.5 mg/mL) for 2 h at 37 °C, and then incubated with H_2O_2 (1 mM) for 6 h. PV, DPV, and PPPs denote phosvitin, partially dephosphorylated phosvitin, and phosvitin oligophosphopeptides, respectively. *, $P < 0.05$, and **, $P < 0.001$, compared with cells treated with H_2O_2 alone. Data are presented as means ± SD in three wells from three separate experiments.

RESULTS AND DISCUSSION

Effects of Phosvitin and Its Derivatives on IL-8 Secretion in H_2O_2-Treated Caco-2 Cells. Caco-2 cells have been reported to produce IL-8 when stimulated by proinflammatory cytokines or oxidative stress (*11*). In the present study, H_2O_2 induced the secretion of IL-8 from Caco-2 cells in a time- and concentration-dependent manner (data not shown). To study the effects of phosvitin and its derivatives on this secretion, Caco-2 cells were pretreated with different concentrations of phosvitin, partially dephosphorylated phosvitin (11% phosphorus retained, DPV), and PPP (0.05, 0.1, and 0.5 mg/mL) or GSH (0.1, 0.5, and 1.0 mM) for 2 h and then stimulated with 1 mM H_2O_2 for 6 h. GSH, a powerful antioxidant, was used as a positive control. As shown in **Figure 1**, DPV and PPP inhibited IL-8 secretion in a concentration-dependent manner, whereas phosvitin did not significantly reduce the secretion. The inhibitory effects of PPP and GSH on IL-8 production were almost identical. Thus, we focused on the protective effects of PPPs against oxidative stress in intestinal epithelial cells.

PPPs were separated into three fractions (PPP-1, PPP-2, and PPP-3) on a Mono Q HR 5/5 anion-exchange column (**Figure 2**). The phosphorus content and amino acid composition (expressed as mole percentages) are listed in **Table 1**. Each fraction was separated according to different contents of phosphorus and Ser. The phosphorus contents were 0, 7.2, and 18.9% in PPP-1, PPP-2, and PPP-3, respectively. PPP-1 contained 9.5 mol % of Ser residues, and PPP-2 contained twice as much Ser as PPP-1. Interestingly, half of the amino acids in PPP-3 comprised Ser residues. PPP-3 was rich in charged basic amino acids such as Arg, Lys, and His. On the other hand, PPP-1 was richer than PPP-2 and PPP-3 in hydrophobic amino acids such as Val, Ile, Leu, and Phe.

We next investigated the inhibitory effects of the three PPP fractions on IL-8 secretion in H_2O_2-treated Caco-2 cells. As

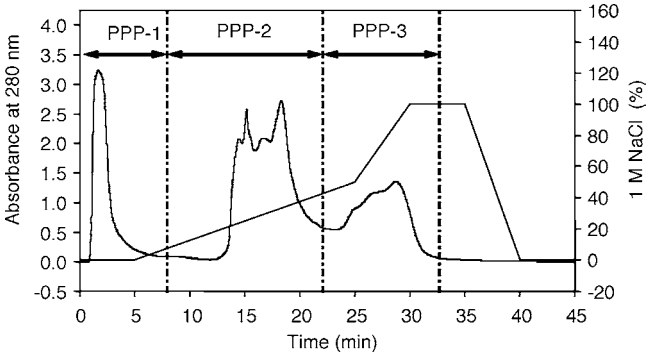

Figure 2. Anion-exchange HPLC analysis of PPPs. Equilibration of the Mono Q HR column and loading of the sample onto the column were performed using 20 mM ammonium bicarbonate (pH 8.0), and elution was performed with a linear gradient of 20 mM ammonium bicarbonate and 1 M sodium chloride, at a flow rate of 1 mL/min.

Table 1. Amino Acid Composition and Phosphorus Content of PPP Fractions

	mol (%) in		
amino acid	PPP-1	PPP-2	PPP-3
Asp	13.6	14.1	9.0
Glu	10.6	12.7	4.4
Ser	9.5	18.4	52.2
Gly	7.3	6.2	4.3
His	3.3	3.3	5.0
Arg	3.4	4.0	7.1
Thr	6.0	5.8	2.0
Ala	8.9	7.7	3.2
Pro	4.8	4.7	1.5
Tyr	2.1	2.1	0.7
Val	8.2	5.5	1.8
Met	1.6	1.4	0.3
Ile	4.4	3.0	0.9
Leu	6.8	5.0	2.1
Phe	3.3	2.6	1.1
Lys	6.0	3.7	4.5
phosphorus (wt %)	0.0	7.2	18.9

shown in **Figure 3**, all PPP fractions decreased the IL-8 secretion in a concentration-dependent manner, with inhibition increasing with the amount of phosphorus. It seems that the phosphorus moieties in PPP fractions have pivotal effects; however phosphoserine itself did not markedly decrease the secretion. Phosvitin, containing 10% phosphorus, did not significantly inhibit IL-8 secretion, and the effect was dramatically enhanced by enzymatic hydrolysis of phosvitin to oligophosphopeptides (**Figure 1**). These suggest that peptide molecular size and amino acid composition contribute to the protective effects against oxidative stress and that phosphorus moieties in oligophosphopeptides could accelerate the antioxidative action. Carnosine (β-alanyl-L-histidine), a dipeptide found in muscle, is known to function physiologically as an antioxidant (*31*), and it also reportedly suppresses IL-8 secretion by H_2O_2-treated Caco-2 cell lines (*32*). The proportion of His residues was higher in PPP-3 than in PPP-1 and PPP-2 (**Table 1**), suggesting that these residues contribute to antioxidative activity against stress in intestinal cells. Future studies should attempt to isolate the active peptides in PPP-3 and identify their amino acid sequences to obtain more structural information of antioxidative-stress peptides.

Molecular masses of PPPs were 1−3 kDa and average chain length was 20.0 as shown in the previous paper (*18*). The precise mechanism by which the oligophosphopeptides penetrate the

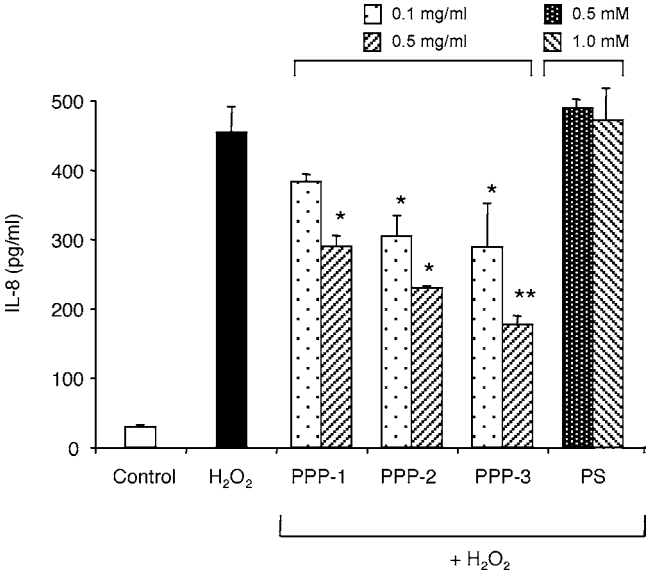

Figure 3. Effects of PPP fractions on IL-8 secretion in H_2O_2-treated Caco-2 cells. PS denotes phosphoserine. *, $P < 0.05$, and **, $P < 0.001$, compared with cells treated with H_2O_2 alone. Data are presented as means ± SD in three wells from two separate experiments.

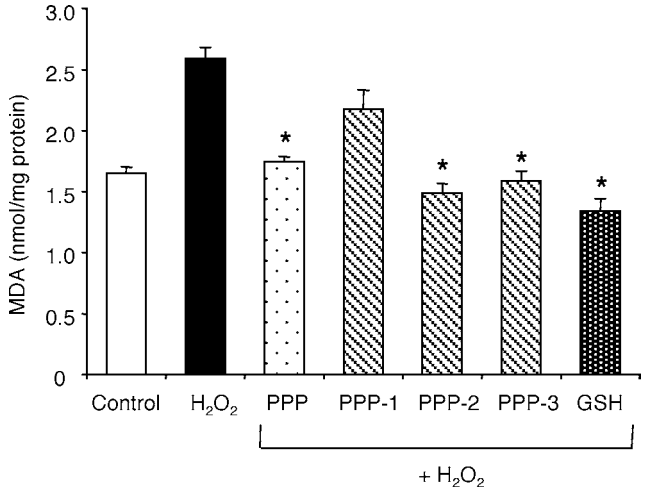

Figure 4. Effects of PPPs and their fractions on MDA levels in H_2O_2-treated Caco-2 cells. Cells were treated with PPPs, PPP fractions (0.5 mg/mL), and GSH (1.0 mM) for 2 h at 37 °C and then incubated with H_2O_2 (1 mM) for 6 h. *, $P < 0.05$, compared with cells treated with H_2O_2 alone. Data are presented as means ± SD in three wells from two separate experiments.

cell transport into the cells in the intact or digested form is under investigation.

Effects of PPPs on Lipid Peroxidation in H_2O_2-Treated Caco-2 Cells. The concentration of intracellular MDA was measured as a second marker of the H_2O_2-induced tissue oxidative stress in the cultured cells. The polyunsaturated fatty acids of cellular membranes are prone to peroxidation by free radicals and oxygen species, which are subsequently degraded to MDA (*33*). Treatment of Caco-2 cells with 1 mM H_2O_2 for 6 h resulted in a significant increase in the MDA concentration (**Figure 4**). MDA production was suppressed by pretreatment with PPP, its fractions PPP-2 and PPP-3, and GSH ($P < 0.05$). In contrast, PPP-1 exerted no significant protective effect. This suggests that only those PPP fractions containing phosphorus protect H_2O_2-induced lipid peroxidation from Caco-2 cells. We have confirmed that phosvitin and PPPs possess a strong radical scavenging activity on DPPH free radical (unpublished data).

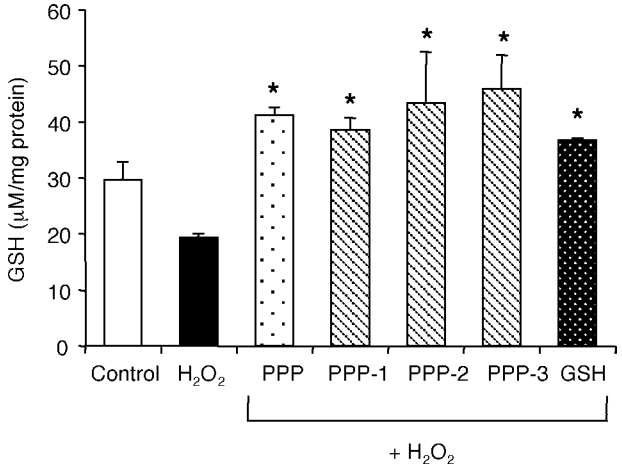

Figure 5. Effects of PPP and its fractions on GSH content in H_2O_2-treated Caco-2 cells. Cells were treated with PPPs, PPP fractions (0.5 mg/mL), and GSH (1 mM) for 2 h at 37 °C and then incubated with H_2O_2 (1 mM) for 6 h. *, $P < 0.05$, compared with cells treated with H_2O_2 alone. Data are presented as means ± SD in three wells from three individual experiments.

The H_2O_2-scavenging activity of PPPs might be responsible for the suppression of IL-8 secretion and MDA production in H_2O_2-treated Caco-2 cells; however, in the present study, phosvitin did not exhibit significantly antioxidative activity against H_2O_2-induced oxidative stress. Thus, the other defense systems such as intracellular antioxidants and antioxidative enzymes may contribute to the mechanism underlying antioxidative stress activity of PPPs.

Effects of PPPs on GSH Levels and GR Activity in H_2O_2-Treated Caco-2 Cells. GSH is the main nonenzymatic antioxidant in cells, and it protects against oxidative damage in systems that scavenge radicals, eliminates lipid peroxidation products, preserves the thiol−disulfide status of proteins, and repairs oxidant damage (*34*). Indeed, it is usually assumed that GSH depletion reflects oxidative damage. As shown in **Figure 5**, the GSH level was lower in H_2O_2-treated Caco-2 cells than in control cells. Pretreatment with PPP and its fractions significantly increased intracellular GSH levels to close to the pretreatment levels. This suggests that the increase in intracellular GSH contributes to the protective effects of PPPs against H_2O_2-induced oxidative stress in Caco-2 cells.

The intracellular ratio of GSH to GSSG (GSH/GSSG) is usually maintained at a relatively high level by a GR-catalyzed recycling pathway. If a tissue is exposed to severe oxidative stress, it may no longer be able to maintain a high GSH/GSSG ratio, leading to the accumulation of GSSG. To determine whether PPPs affect the intracellular redox balance, we further investigated the effects of PPPs and its fractions on GR activity in H_2O_2-treated Caco-2 cells. As shown in **Figure 6**, treatment with PPP-2 and PPP-3 prior to the induction of oxidative stress markedly enhanced the GR activity in Caco-2 cells ($P < 0.05$), whereas PPP, PPP-1, and GSH produced only a nonsignificant increase. This result suggests that PPP-2 and PPP-3 can maintain a high intracellular GSH/GSSG ratio by enhancing GR activity.

In the present study, PPP exhibited antioxidative effects on stress by suppressing the inflammatory response and acting as a lipid peroxidation inhibitor. In addition, these oligophosphopeptides maintained a high intracellular GSH concentration. The first mechanisms by which PPPs could restore oxidative-stress-mediated cellular GSH depletion is via activation of GR activity, which accelerates the conversion of GSSG to GSH. Second, PPPs might up-regulate GSH biosynthesis. As shown in **Figure**

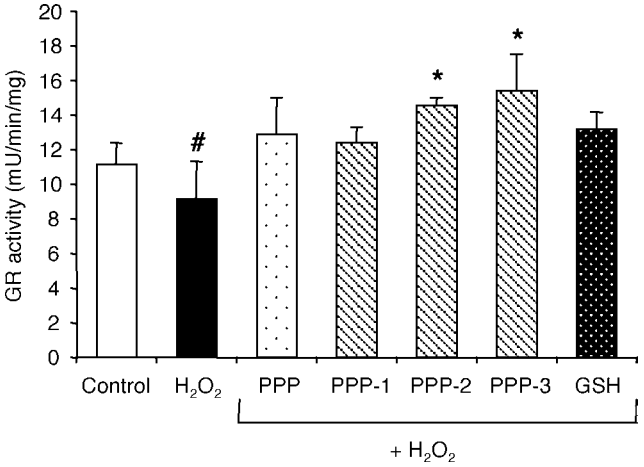

Figure 6. Changes in GR activity in H_2O_2-treated Caco-2 cells. The cells were treated with PPPs, PPP fractions (0.5 mg/mL), and GSH (1.0 mM) for 2 h at 37 °C prior to incubation with 1 mM H_2O_2 for 6 h. #, $P < 0.05$, compared with control cells. *, $P < 0.05$, compared with cells treated with H_2O_2 alone. The bars represent ± SD of three wells. Results are representative of two experiments.

5, pretreatment with PPPs significantly increased GSH levels of H_2O_2-treated cells, and the GSH levels of PPPs-pretreated cells were higher than that of the control cells. The cellular GSH level is increased by up-regulation of the γ-glutamylcysteine synthetase gene (*35*). Curcumin is a polyphenol of the curcuminoid family that reportedly exhibits free radical scavenging activity as an antioxidant (*36*, *37*). Biswas et al. (*38*) have demonstrated that curcumin protects alveolar epithelial cells (A549) from H_2O_2-mediated depletion of GSH levels and inflammatory events, including IL-8 secretion, by up-regulation of GSH biosynthesis. In the present study, we showed that PPPs increase GR activity, but we did not investigate their effects on the regulation of GSH synthesis. Therefore, the mechanism underlying the marked increase in intracellular GSH levels needs to be further explored.

In conclusion, the present study demonstrates that oligophosphopeptides from hen egg yolk exert protective effects against tissue oxidative stress in intestinal epithelial cells.

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