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Reduction of hexavalent chromium by digested oat bran proteins

Apollinaire Tsopmo [a,b,*], Qing Gao [b], Morooj M. Baakdah [a]

[a] Food Science and Nutrition Program, Department of Chemistry, Carleton University, 1125 Colonel By Drive, K1S 5B6 Ottawa, ON, Canada
[b] Institute of Biochemistry, Carleton University, 1125 Colonel By Drive, K1S 5B6 Ottawa, ON, Canada

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ABSTRACT

Hexavalent chromium (Cr(VI)) is associated with an increase in oxidative stress, such as DNA, lipid and protein oxidations. This study investigated, for the first time, the ability of digested food proteins to reduce Cr(VI) and scavenge free radicals. Protamex, an endopeptidase was used to digest oat bran protein isolates for 1, 2, 3, and 4 h. The hydrolysates (1 mg/ml) showed a maximum Cr(VI)-reducing activity of 5.4 ± 0.4% and 44.6% at pH 7.4 and 3.0, respectively. The difference might be due to charge or conformation changes depending on the pH. The 2 h hydrolysate possessed the highest $O_2^{-\cdot}$ inhibition activity (57.4 ± 5.1%), while the 1 h hydrolysate had the highest $HO^{\cdot}$ inhibition (11.6 ± 0.6%). A correlation (R^2 = 0.82) was observed between the $O_2^{-\cdot}$ scavenging activities of hydrolysates and their Cr(VI)-reducing activities at pH 3.0 only. Further investigations of the digested oat bran proteins are required to determine their ability to reduce Cr(VI) oxidative stress damage *in vivo*.

1. Introduction

Metals play important roles in a wide variety of biological processes in living systems. The breakdown of metal-ion homeostasis can lead to uncontrolled metal-mediated formation of deleterious free radicals and subsequent modifications of DNA bases, enhanced lipid peroxidation and altered calcium and sulphydryl homeostasis (Valko et al., 2007). Some of the redox-active metals closely linked to the generation of free radicals in living systems are iron, copper, cobalt and chromium (Mates, Segura, Alonso, & Marquez, 2008).

Chromium (Cr) exists in several oxidation states, with the most common and stable forms being trivalent (Cr(III)) and hexavalent (Cr(VI)). The latter is widely used in diverse industries, including pigment manufacturing, painting, metal plating and leather tanning (Wang, Xing, Shen, Zhu, & Xu, 2006). Hexavalent chromium and its derived compounds are of concern because of their toxicity (i.e. carcinogen). Subcutaneous administration of Cr(VI) to rats was reported to cause severe progressive proteinuria, increase blood urea nitrogen and creatinine, and hepatic lipid peroxides formation (Kim & Na, 1991). Related studies also demonstrated that a single subcutaneous injection of Cr(VI) to rats induced renal damages, which were associated with increases in nitrotyrosine, protein carbonyls and urinary excretion of proteins (Gumbleton & Nicholls, 1988). An increase in markers of oxidative stress, including hepatic mitochondrial and microsomal lipid peroxides, urinary malondialdehyde, formaldehyde, acetaldehyde, acetone and propionaldehyde, were found in rats that received orally water containing Cr(VI) (Bagchi, Hassoun, Bagchi, Muldoon, & Stohs, 1995). In chronic myelogenous leukemic K562 and J774A.1 murine macrophage cells, Cr(VI) enhanced the production of reactive oxygen species that resulted in oxidative tissue and DNA damages (Bagchi, Bagchi, & Stohs, 2001). Treatment of human lung epithelial cells with Cr(VI) caused apoptosis (Ye et al., 1999). In humans, an increased risk of respiratory cancers were found in workers occupationally exposed to Cr(VI) compounds (Costa, 1997), while higher peripheral lymphocytes, DNA strand breakage and urinary lipid peroxidation products were found in chrome-plating workers compared to control university workers (Gambelunghe et al., 2003; Goulart, Batoreu, Rodrigues, Laires, & Rueff, 2005).

Amongst the most common forms of chromium, trivalent chromium Cr(III) is not a substrate of the cellular anion transport system, is poorly absorbed from the gastrointestinal tract and is unable to cross the cell membrane (Costa, 1997; Salnikow & Zhitkovich, 2008). There is, however, a possibility that small amounts of Cr(III) can enter the cell through the energy intensive process of pinocytosis (Jomova & Valko, 2011). Compared to Cr(III), the absorption of Cr(VI) is higher because it is often present in a tetrahedral coordination, such as oxyanions (e.g. CrO_4^{2-}), that can enter many types of mammalian cells under physiological conditions via non-specific anion transporters (Stohs & Bagchi, 1995). Both Cr(VI) and Cr(III) are involved in redox cycling and the production of reactive oxygen species (O'Flaherty, 1996). Once taken up, Cr(VI) is rapidly reduced to the pentavalent chromium (Cr(V)), tetravalent chromium (Cr(IV)) and then to Cr(III) by non-enzymatic (e.g. ascorbic acid, cysteine, reduced glutathione) and enzymatic (e.g.

* Corresponding author at: Food Science and Nutrition Program, Department of Chemistry, Carleton University, 1125 Colonel By Drive, K1S 5B6 Ottawa, ON, Canada. Tel.: +1 6135202600x3122 ; fax: +1 6135203749.
E-mail address: apollinaire_tsopmo@carleton.ca (A. Tsopmo).

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glutathione reductase) reductants (Nickens, Patierno, & Ceryak, 2010). Intracellularly, Cr(VI) can also be reduced by hydrogen peroxide with concomitant production of reactive oxygen species (ROS) and cellular damages (O'Brien, Ceryak & Patierno, 2003).

Although some studies have been performed on the role of cysteine and GSH in the reduction of Cr(VI) and mitigation of its cytotoxicity (Lay & Levina, 1996; Quievryn, Goulart, Messer, & Zhitkovich, 2001), no method has been developed for *in vitro* testing of digested food proteins or phytochemicals. The objective of this study was then to develop a procedure to measure the Cr(VI) reducing capacity of different digested oat bran proteins and also to measure the superoxide anion and hydroxyl radicals scavenging activities of the protein digests.

2. Methodology

2.1. Materials and chemicals

Medium oat bran flour (item number 112-001) was supplied by Can-Oat Milling Inc. (Portage La Prairie, MB, Canada). Viscozyme L® 100 Fungal Beta Glucanase (FBG)/g, potassium dichromate, pyrogallol, Tris–HCl, ethylene diamine tetra-acetic (EDTA), sodium phosphate monobasic dihydrate, citric acid monohydrate, 1,10-phenanthraline, cysteine, glutathione and mono- and dibasic potassium phosphates were obtained from Sigma–Aldrich (Oakville, ON, Canada). Methanol, hexane, hydrogen peroxide and ferrous sulfate heptahydrate were purchased from Fisher Scientific Co. (Nepean, ON, Canada). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) was obtained from Wako Chemical (Richmond, VA, USA). All spectrophotometric measurements were performed on the BioTek® Epoch™ UV–Vis microplate reader controlled by Gen5™ data analysis software while incubations were done on a MaxQ™ 5000 incubator shaker model (Fisher Scientific, Nepean, ON).

2.2. Extraction of proteins

The medium oat bran flour sample (3×100 g grammes) was defatted by mixing with hexane (1:3 w/v) and stirring for 1 h at room temperature in a fume hood using a 10×10 inch magnetic stirrer plate (VWR International, Mont-Royal, Quebec). Hexane containing the fat was removed by filtration on Whatman paper No. 1. The defatted bran was left to dry overnight in a fume hood, suspended in water 1:10 (w/v) ratio and mixed to obtain a homogeneous slurry whose pH was adjusted to 4.5. Viscozyme, a multienzyme complex that was demonstrated to decrease the viscosity of the slurry and improve protein extraction (Jodayree, Smith, & Tsopmo, 2012), was added at a ratio of 3 FBG/g of defatted bran, followed by incubation at 45 °C, 150 rpm for 2 h. At the end of the incubation, the pH was adjusted to 9.5 with 2 M NaOH solution and further incubated for 30 min. The alkaline solution was cooled down and centrifuged at 2500*g*, for 20 min and 4 °C. To obtain the protein isolate, the supernatant was collected followed by adjustment to pH 4.0 and centrifugation at 10,000*g* for 40 min at 4 °C. The obtained protein isolate (precipitate) was washed with water (pH 4.0) and centrifuged for 30 min at 1100*g*. Finally, the isolate was dissolved in water, adjusted to pH 7.0 and freeze dried.

2.3. Hydrolysis of proteins with protamex

The oat bran protein isolate (800 mg each) was transferred to eight different tubes and suspended in water 1:12 ratio. The pH was adjusted to 6.5 with 1 M NaOH and 1 M HCl followed by the addition of protamex at an enzyme ratio of 50 mg/g of protein isolate. To perform the hydrolysis, samples were incubated at 50 °C and 150 rpm. Two samples were removed at 1, 2, 3 and 4 h, respectively. At the end of each incubation time, the enzyme was inactivated by heating at 90 °C for 10 min and the tubes were allowed to cool by putting them in a refrigerator at 4 °C. Supernatants were collected after centrifugation at 5000*g* for 25 min to remove enzymes and non-digested proteins, freeze dried and stored and at −20 °C until further analyses.

2.4. Chromium (VI) reducing activity

Solutions of Cr(VI) in the form of potassium dichromate ($K_2Cr_2O_7$, 10^{-4} to 10×10^{-4} M) were prepared in 0.1 M phosphate buffer (pH 7.4) and in 0.12 M citric acid–phosphate buffer (pH 3.0). The citric acid–phosphate buffer was made by mixing 79.5 ml of 0.1 M citric acid monohydrate and 20.6 ml of 0.2 M sodium phosphate dihydrate. The spectra were recorded from 200 to 800 nm in a 1:1 mixture of Cr(VI) solutions with respective buffers. Cysteine and GSH 0.25×10^{-3}, 0.5×10^{-3}, 10^{-3} and 2×10^{-3} M were prepared in both phosphate and citric acid–phosphate buffers for analysis in a neutral and acidic environment. To measure the Cr(VI) reducing activity, 100 μl of Cr(VI) was mixed with 100 μl of cysteine or GSH in a 96-well clear microplate, sealed with film and incubated at 37 °C for 1 h (120 rpm). The absorbance was read at 370 nm. The control was 100 μl Cr(VI) plus 100 μl of buffer, while the blank was 200 μl of buffer.

Protamex digested oat bran proteins (2.5 and 5 mg/ml) were dissolved in 0.1 M phosphate buffer (pH 7.4) and in 0.12 M citric acid–phosphate buffer (pH 3.0), treated with Cr(VI) (2×10^{-4} or 5×10^{-4} M) and processed as above. This time, sample blanks, made by adding 100 μl of digested proteins to 100 μl of buffer, were included because oat bran protein hydrolysates have slight absorbances at 370 nm. Cys and GSH had no absorbance at 370 nm. The positive control was GSH (2.5 and 5 mg/ml). The chromium VI reducing activity was calculated as follows:

$$\text{Cr(VI) reducing activity } (\%) = \frac{\text{Ac} - (\text{As} - \text{Asb})}{\text{Ac}} \times 100$$

where: Ac: absorbance control (Cr(VI) + buffer); As: absorbance sample (digested proteins + Cr(VI)); Asb: absorbance sample blank (digested protein + buffer).

2.5. Superoxide scavenging activity

The superoxide scavenging activity of the digested oat proteins and glutathione (positive control) was measured as previous reported (Alrahmany & Tsopmo, 2012). Briefly, samples and GSH were dissolved in 50 mM Tris–HCl buffer containing 1 mM EDTA, at pH 8.3 to a final concentration of 1 mg/ml. To run the assay, 80 μl of each digested oat bran protein or GSH control was mixed with 80 μl of buffer in a 96-well clear round bottom microplate in darkness. Then, 70 μl of 1.5 mM pyrogallol dissolved in 10 mM HCl was added. The reaction rate (ΔA/min) was measured immediately at 420 nm for 4 min at room temperature on a BioTeK microplate reader. The buffer was used as a negative control. The superoxide scavenging activity was calculated using the following equation:

$$\text{Scavenging activity } (\%) = ((\Delta A/\text{min})c - (\Delta A/\text{min})s)/(\Delta A/\text{min})c \times 100$$

2.6. Hydroxyl radical ($HO^{\cdot}$) scavenging assay

The $HO^{\cdot}$ scavenging assay was modified on the basis of a reported procedure (Alrahmany & Tsopmo, 2012). Protamex digested oat proteins and glutathione (positive control) were prepared in 0.75 mM potassium phosphate buffer at pH 7.4. Then