Structure-function of hydroxyl radical scavenging and chromium-VI reducing cysteine-tripeptides derived from rye secalin

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Abstract

This study evaluated for the first time hydroxyl radical scavenging and chromium VI (Cr(VI) reducing properties of four cysteine containing peptides derived from rye secalin. Density functional theory (DFT) calculations were performed to determine the antioxidation mechanism and the effect of residue order. The peptides tested (CQV, QCA, QVC, QCV) were obtained from *in silico* digestion of rye secalin with Proteinase-K and selected because they contained thiol, a known redox functional group. It was found that at pH 7.4, CQV had the highest Cr(VI) reducing activity (76 %) followed by QCA and QCV (30.8 and 25.5 %, respectively). QVC and GSH had similar but lower activities (11.3 and 11.7%). At pH 3.0, CQV and QCV were found to be less active than the other two peptides. In the hydroxyl radical scavenging assay, CQV had the highest activity with 28.9 ± 1.3 % inhibition of the formation of HO• radicals compared to 19.0 – 13.6% for other peptides. The highest reactivity of CQV with Cr(VI) under neutral conditions was due to the proximity of thiol and amine of glutamine that allowed the formation of a transition state that facilitated the reduction. Cysteine at the N-terminal was important for both the reduction of chromium (pH 7.4) and the HO• scavenging activity because the S-H bond at that position was found by the DFT analysis to have the lowest bond dissociation energy.

Keywords: antioxidant, density functional theory, peptides, hexavalent chromium

1. INTRODUCTION

The toxicity of hexavalent chromium (Cr(VI)) is due to the formation of Cr-DNA adducts as well as oxidised biomolecules because of excess production of hydroxyl radicals (Ye et al., 1999). Small molecules such as ascorbate and lipoic acid can reduce the toxicity of Cr(VI) as a result of radical scavenging activity and rapid reduction of Cr(VI) (Sugiyama, Tsuzuki, & Ogura, 1991; Zhitkovich, 2011). Other reducers are cysteine and glutathione (Wiegand, Ottenwälder, & Bolt, 1984). Recently hydrolyzed oat proteins were demonstrated to possess Cr(VI) reducing activity (Tsopmo, Gao, & Baakdah, 2014) but other hydrolysates or sulfur containing peptides have not been investigated. Cereal storage proteins (i.e. prolamins) are characterized by unusual amino acid compositions with high proline and amide nitrogen. Their trivial names are secalins (rye), gliadins (wheat), and hordeins (barley). The nutritional quality of this group of proteins is low because of less amount of the essential amino acid lysine. Studies have used proteases to produce hydrolysates or peptides with activities such as antioxidant and anti-hypertensive from wheat and barley (Bamdad, Wu, & Chen, 2011). Although, amino acids like Tyr, Trp, His, Cys can contribute to the antioxidant activity of peptides, there is only scanty information on structure activity relationships of peptides as most works have focussed on polyphenols (Borgohain, Guha, Pratihar, & Handique, 2015; Cai, Chen, Xie, Zhang, & Hou, 2014). The discovery of novel bioactive peptides can then be accelerated by investigating their structure activity relationships using computational methods. Previous works have shown that parameters such as bond dissociation energy, ionisation potential, steric hindrance, hydrogen bonding energy, proton dissociation enthalpy, and proton affinity can be related to functionalities of

molecules (Xue, Zheng, An, Dou, & Liu, 2014; Yehye et al., 2015). A computational analysis based on the density functional theory (DFT) was used to calculate and rationalize the antioxidant activity of thiosemicarbazide derivatives (Nazarbahjat et al., 2014). DFT was also used to show that the reaction of aromatic amines with alkoxyl radicals in aprotic solvents depended on the N-H bond dissociation energy and the stability of the of aminyl radical (Lucarini et al., 1999). Rye is of lesser economic importance compared to other cereals and the identification of bioactive peptides derived from proteins can increased its value (Shewry, Kreis, Burgess, Parmar, & Miflin, 1983). Furthermore, there is no computational study regarding the activity of rye peptides. The aim of this study was then to investigate the ability of peptides derived from a rye secalin protein to reduce chromium VI and scavenge hydroxyl radicals that generally accompany this reduction. It also aimed to determine molecular descriptors responsible for the activity.

2. MATERIALS AND METHODS

2.1. Chemicals and materials

Potassium phosphate monobasic, glutathione (GSH), sodium phosphate monobasic dihydrate, citric acid monobasic, potassium dichromate, ferrous sulfate hepahydrate ($FeSO_4 \cdot 7H_2O$), 1,10-phenanthronine and hydrogen peroxide were purchased from Sigma-Aldrich Co (Oakville, ON, Canada). Microplate spectrophotometer model Epoch™ controlled by Gen5™ data analysis software (Fisher Scientific, Nepean, ON) was used to analyse samples. Rye secalin (UniProtKB/ TrEMBL i.d. Q9FR41) was digested using proteinase K through a simulated digestion using UniProt PeptideCutter available at http://web.expasy.org/cgi-bin/peptide_cutter/peptidecutter.pl?

Q9FR41. Two tripeptides containing cysteine were identified, CQV and QCA. These were then synthesized at 95% purity by GenScript Inc. (Piscataway, NJ, USA). The cysteine positions were also changed to obtain, QCV and QVC.

2.2. Chromium reduction assays

Chromium (VI) reduction assays were performed based on a reported procedure at neutral and acidic conditions (Tsopmo et al., 2014). At pH 7.4, 0.1 M phosphate buffer (pH 7.4) was used to make 0.2 mM of a Cr(VI) solution from potassium dichromate $K_2Cr_2O_7$, 5 mM of peptides (CQV, QCV, QVC, QCA) and control (GSH). At pH 3.0, citric acid phosphate buffer (0.12 M, pH 3.0) was used to make 1 mM of $K_2Cr_2O_7$, and 1 mM solution of peptides and control. For analysis, 100 µL of each peptide was transferred in triplicate into 96-well clear microplate followed by addition of 100 µL of Cr(VI). The control contained 100 µL of buffer instead of sample. The plate was sealed incubated at 37 ^oC for 1 h. Readings were recorded after at 30 min at 370 nm. A kinetic assay was done to determine the reaction rate of each peptide. Concentrations were 0.2, 0.4, 0.6, 0.8 and 1.0 mM at pH 3.0 while they were 1.0, 2.0, 3.0, 4.0 and 5.0 mM at pH 7.4. Data were recorded at 15 sec intervals for 60 min at 370 nm. Pseudo-first-order rate constants were derived plots of $ln(A_{obs} - A_{\infty})$ versus time

2.3. Hydroxyl radical scavenging assay

The assay was performed based the generation of HO• radicals from a Fenton reaction between ferrous ions and hydrogen peroxide (Vanvi & Tsopmo, 2016). Peptides and glutathione were prepared at 1.6 and 3.2 mM in phosphate buffer (0.1 M, pH 7.4). In a 96-well plate, the following

were added in quadruplicate: peptide (50 µL), 1,10-Phenanthroline (50 µL, 3 mM) and iron(II) sulfate (50 µL, 3 mM). To initiate the reaction 50 µL of 0.03% hydrogen peroxide (H_2O_2) was added and the plate was incubated at 37 °C for one hour before absorbance reading at 536 nm. The percentage inhibition was calculated as reported (Alrahmany & Tsopmo, 2012).

2.4. Structre-function study using density functionaltheory

Calculations were performed using GAMESS-US (Schmidt et al., 1993), with the 6-311G(d,p) basis set and the M06-2X functional (Zhao & Truhlar, 2008). Antioxidant molecules can function either through the homolytic bond dissociation that provides a hydrogen to saturate the oxidizing species or by giving an electron to as well saturate the oxidant. In order to determine which of two mechanisms was dominant, we evaluated the thiol (-SH) bond dissociation energies (BDE) and ionization potentials (IPs) each molecule (CQV, QCV, QVC, QCA, and related peptides). Using equations (1) and (2), respectively.

Structural optimizations were carried out for all ionic and neutral, radical and closed-shell species in the two equations. The reported bond dissociation energies and ionization potentials include the contributions of zero-point energies.

2.5. Statistical analysis

All results are presented as mean ± standard deviation from replicates. (n = 4). One way ANOVA was used, differences between means were calculated using Tukey's test. Statistical significance

was set to $p < 0.05$. All statistics were completed with IBM SPSS software IBM SPSS version 22 (Armonk, NY, USA). Pearson correlation coefficients were used to determine relationships between data.

3. RESULTS AND DISCUSSION

Computational or *in silico* methods are useful tools in structure-functionality relationships analysis of peptides derived from food proteins. They have been used to generate thousands of small peptides (2 to 6 amino acids) from proteins of 15 food commodities many of which, were predicted to possess potent angiotensin converting enzyme (ACE)-inhibiting properties (i.e. $IC_{50} < 10$ μM) (Gu, Majumder, & Wu, 2011). They have also been used to predict the antioxidant activity of peptides from cereal RuBisCO proteins (Je, Cho, Gong, & Udenigwe, 2015). Secalins are major storage proteins present in rye. During proteolytic digestion, the related wheat storage proteins (e.g. gliadins) can release polypeptides (rich in proline and glutamine) believed to be responsible for the auto-immune response in celiac enteropathy (De Angelis et al., 2006). However, they can also release bioactive peptides as a decamer peptide from wheat prolamins that prevented the activation of T-cell activation (De Vita et al., 2012). In this study, Rye secalin was in-silico digested with Proteinase K, a protease with broad specificity but also with predominant cleavage sites at bonds adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha amino groups (Ebeling et al., 1974). Two tripeptides CQV and QCA were selected as previous study has shown redox properties of thiol groups are important for metal reducing and antioxidant properties (Ramdon, Dixon, & Dasgupta, 2002;

Wiegand et al., 1984). The position of cysteine was varied for structure-function relationship investigations.

3.1. Chromium VI reducing activity

The Cr(VI) reducing properties of the four peptides and GSH were determined at both neutral and acidic environment to reflect what may happen in vivo. The concentration used was 1.0 mM Cr(VI) in acidic condition compared to 0.2 mM for neutral environment and this is because of difference in stabilities of dichromate at both conditions (Ramdon et al., 2002; Tsopmo et al., 2014). Data showed that at pH 7.4 (Figure 1A), CQV was the most potent and reduced Cr(VI) by 73 ± 2.6%. It was followed QCA and QCV with a reduction of 30.8 ± 2.8% and 25.5 ± 2.0%, respectively. The least active was QVC, 11.7 ± 2.8 and this was similar to the activity of GSH. The Cr(VI) reducing activity found for GSH was similar to that a previous study (Tsopmo et al., 2014). Cysteine at the N-terminal of CQV enhanced the activity by almost 3-fold compared to the one with cysteine at the C-terminal. It appeared that the proximity of the thiol group and the amide on the side chain of glutamine might have facilitated the formation of thiolate-Cr(VI) complex and subsequent reduction according to the general scheme proposed for some thiols (O'Brien, Wang, & Wyatt, 1992).

In acidic solution (Figure 1A), QVC, QCA and GSH had similar reducing 33.6 – 37.0% ($P > 0.05$) however, it was higher ($P < 0.05$) compared to the activity of CQV (24.5 ± 2.6) and QCV (24.7 ± 2.8). Peptide CQV had the highest activity at neutral pH but not true at the acidic pH. Recently, digested proteins from oat in the presence of Cr(VI) were found to behave differently as well depending on the pH of solutions (Tsopmo et al., 2014). Literature data

showed that the reduction of chromate by thiol-containing molecules initially proceeded via formation of thiolate-Cr(VI) complex which may then at low pH decompose by acid-catalyzed pathway. However, in neutral solution, a second mole of thiol compound is useful for the reduction (Lay & Levina, 1996). Overall, tripeptides have stronger Cr(VI) reducing capacity at pH 3.0 (sample concentrations 5-fold lower) and this can be explained by the stronger oxidizing power of Cr(VI) under acidic conditions as reported in a related study (Ramdon, Dixon & Dasgupta, 2002).

The effect of peptide concentration (1 – 10 mM at pH 7.4 and 0.2 – 1.0 mM at pH 3.0) on the reduction of dichromate was determined. The reduction followed a pseudo-first order reaction at 370 nm and the observed rate (k_{obs}) was obtained from $\ln(A - A_\infty)$ versus time and this was in agreement with previous studies on Cr(VI) reactions with glutathione, mercapto-succinic acid or propionic acid (Lay & Levina, 1996; O'Brien et al., 1992; Ramdon et al., 2002). The rate varied linearly with the concentration of peptide. At neutral environment (5 mM sample), the rate of reduction by CQV ($k_{obs} = 0.044\ s^{-1}$) was at least 3.6-fold higher compared to the rate of other peptides ($k_{obs} = 0.012 – 0.004\ s^{-1}$). Two molecules of peptides are required to form the thiolate intermediate and two other molecules are needed for the reduction of Cr(VI) to Cr(III) and during both steps, a free amine group can participate in the reduction process. The highest rate for CQV can then be attributed to the formation of another intermediate state with the amine located on the side chain of glutamine because of its relative proximity with SH-group compared to other peptides. This is supported by a previous study that showed the formation of a 7-membered ring transition state in the reduction of chromate by 2-mercaptosuccinic acid (Ramdon et al., 2002). Amongst the tested peptides, QVC had the lowest reduction rate at pH

7.4. In the acidic environment, the observed rate for QVC and QCV were similar at the lowest concentration (0.2 mM), however as the concentration increased QVC react slowly compared to not only QVC but also to the two other peptides and as a consequence possessed the lowest rate at 1 mM (k_{obs} = 0.009 s^{-1}), while the other three peptides had comparable rates ((k_{obs} = 0.011 – 0.0134 s^{-1}). Differences in reactivity of the same peptides at different pH can be due to modification of the conformation as it had been reported that variation in acidity resulted in significant changes of reaction rate constants probably due to the deprotonation of reductants, shapes, and net-charge differences (O'Brien et al., 1992)..

3.2. Hydroxyl radical scavenging activity of tripeptides

The reduction of Cr(VI) generates thiyl, superoxide anion and hydroxyl radicals which are involved in chromium genotoxicity (O'Brien & Kortenkamp, 1995). In addition to their reducing properties, the hydroxyl radicals (HO•) scavenging activity of peptides is therefore important to prevent lipids, proteins and DNA damages (Nickens, Patierno & Ceryak, 2010). It was found (Figure 1B) that CQV was the most active and inhibited by 28.9 ± 1.3% the generation of HO• radicals. It was followed by QVC with 19.0 ± 1.1% inhibition. QCV and QCA had similar activities and prevented the formation of HO• radicals by 15.0 ± 1.2 and 13.6 ± 1.0%, respectively. Control GSH has the lowest activity (8.6 ± 0.8%). CQV with the highest HO• activity was also the strongest Cr(VI) reducing peptide at pH 7.4. Recently, oat bran proteins digested with different concentrations of protamex were reported to inhibit the formation of HO• radical by 3.7 – 11.6% (Tsopmo et al., 2014) which is lower that the activity of tripeptides investigated in this study. The fact that tripeptides had better HO• activities than GSH is an

indication that they may spare GSH use and reduce oxidative stress resulting from Cr(VI) exposure. The scavenging activity of tripeptides was dependent on the location of each amino acid on the sequence. Factors such as electronic properties, steric hindrance, hydrophobicity, and hydrogen bonding can also affect the activity of peptides. It was reported that differences in ferric-reducing antioxidant power assay of related beta-lactoglobulin tripeptides were due to a combination of those factors (Tian et al., 2015). For example the reducing property of peptide CAQ (13.6 mM Fe/mole) was almost haft of ACQ (23.8 mM Fe/mole) value while QCL reducing activity was 49.4 mM Fe/mole compared 10.0 mM Fe/mole to QCH (Tian et al., 2015). The sulfur containing compound *S*-allylcysteine from garlic was reported to protect LLC-PK_1 cells from potassium dichromate-induced toxicity through its ability to scavenge $HO^{\bullet}$ radicals (Medina-Campos et al., 2007).

3.3. Structure-function relationship

In this work, *ab initio* quantum chemistry methods were used to determine the mechanism of hydroxyl radical ($HO^{\bullet}$) scavenging and hexavalent chromium reducing peptides as well as predict the suitability of other isomers as candidates for future investigation. The activity of antioxidants can be examined by homolytic cleavage of thiol bonds (-SH) (e.g. proton transfer) or by ionisation (electron transfer) (Nazarbahjat et al., 2014; Xue et al., 2014). In order to determine which of these processes was dominant, thiols (-SH) bond dissociation energies (BDEs) and ionization potentials (IPs) of the five tested antioxidant peptides were calculated (Table 1). The $HO^{\bullet}$ activities of CQV, QCV, QVC, QCA, and glutathione had an almost perfect negative correlation with BDE (R^2 = 0.99, Figure 3A) but a less perfect negative one with IP (R^2

= 0.93, Figure 3B). Therefore, it is believed that the homolytic S–H bond cleavage was the dominant mechanism for the antioxidant activity.

To determine the effect of sequence on the HO• scavenging activity, the BDE was calculated for each tripeptide formed by a unique combination of valine, cysteine and glutamine; or alanine, cysteine and glutamine. The additional eight structures are shown in Figure 2, and the results are summarized in Table 2, pairs P1 to P6. The change in alkyl group had a significant impact on BDE, with the largest shift found between VQC and AQC (Δ_{BDE} = 6.47 KJ/mole). In an attempt to further lower the BDEs, amino acids with electron-donating side chains like phenylalanine (F) and aspartic acid (D) were introduced while maintaining cysteine (C) and alanine (A). The four examined structures (CAF, CFA, CAD, CDA) are shown in Figure 2 and their calculated BDEs are given in Table 2 (pairs P7 – P10), in comparison with CQV, the tripeptide with the lowest BDE among the tested series. CDA was found to have lower BDE than CQV. Actually, it has the lowest the BDE among all structures that are calculated, and could be a promising antioxidant.

In the very end, we calculated HO• scavenging activities of all structures in Figure 2 using the trend line equation (y = -0.376x + 345.11) from the scattered plot of experimental HO• activities of the five tested tripeptides and their BDEs. The predicted HO• data are plotted against the calculated BDEs, along with the data from tested peptides (CQV, QCA, QVC, QCV) and the control glutathione (Glut) (Figure 3C). The negative inhibition percentage of CAD should not be taken seriously, as it arises from the linearity of the trend line equation. This figure (3C) clearly illustrates the relationship between antioxidant functions and bond energies of all considered tripeptides. It is evident based on this model that CDA is outstanding and shall be a target for

future investigations. On the other hand, AQC, VQC, and CAD are predicted to be weaker hydroxyl radical scavengers amongst the tripeptides.

CONCLUSION

Tripeptides investigated in this study effectively converted hexavalent chromium to the stable and beneficial trivalent chromium. At the same time, they scavenged hydroxyl radicals that are responsible for oxidative damage to macromolecules. DFT calculations indicate that the homolytic dissociation of the thiol bond is the dominant mechanism for the hydroxyl radical activity. Bond dissociation energies of a series of structures other than those tested, showed that peptide CAD is a good candidate for future investigation.

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