UNIVERSITY OF HELSINKI

Department of Food and Environmental Sciences

EKT Series 1648

OXIDATION OF ALPHA-LACTALBUMIN PEPTIDES WITH AND WITHOUT PROCYANIDINS

Xin Zeng

Helsinki 2014
PREFACE

This study was performed at the Department of Food and Environmental Sciences, Food Chemistry Division, at the University of Helsinki.

First of all, I would like to give my deepest gratitude to Professor Marina Heinonen for leading me to this exciting field of protein oxidation. As my supervisor, you are always prepared for all of my questions concerning every aspect of the work. I am so impressed by your patience and kindness. Your inspiring words motivated me to complete the thesis with efficiency. Thank you also for the valuable comments during the writing process of the thesis.

Especial thanks go to my supervisor Tuuli Koivumaki for her guidance during the work. You instructed me with good laboratory practices and guided me through the experiment. I appreciate your patient help very much since summer training. Another supervisor Bei Wang deserves my sincere thanks. You are more of a friend to me, helping me with the analysis of the data and comforting me when I was down. I am also thankful for Miikka Olin, Göker Gürbüz, Bhawani Chamлагain, Petri Kylli, Paula Kahila and Lingdai Liu who gave technical supports in the lab work.

I wish to thank the coordinator of MSc Food program Ms Tiina Naskali, who helped me through the master study. Moreover, I would like to thank all the people in the coffee room for providing a comfortable environment for dinner.

At last, I would like to give my earnest gratitude to my parents for their spiritual support throughout my life. Millions of thanks are far less than what they had given me and never can I pay back. The only thing I can do is to keep that love deep in heart quietly and eternally.

Helsinki, May 2014

Xin Zeng
The literature review part describes a whey protein, α-lactalbumin (ALA) and its oxidation, aronia (Aronia melanocarpa) procyanidins including their chemistry and analysis, as well as interactions between protein and plant phenolics. The aim of the thesis was to study the oxidation of tryptic peptides of ALA with and without procyanidins.

Fractions containing both procyanidin dimers and trimers were collected from aronia juice proanthocyanidins with preparative-HPLC thereafter identified and quantified with UHPLC and NP-HPLC. ALA was digested by modified trypsin and the subsequent peptides were fractionated with preparative-HPLC. The selected peptides were WLAHKALC (m/z 936) and LDQWLCEK (m/z 1034), containing specific amino acids prone to oxidation. The oxidation of peptides with and without procyanidins was catalysed by ascorbic acid, H$_2$O$_2$ and FeCl$_3$, and lasted for 14 days. Oxidation was monitored with LC-MS using samples collected on days 0, 1, 7 and 14. For peptide-phenolic interaction studies, peptide WLAHKALC (m/z 936) was incubated with procyanidin B2 standard (dimer) and peptide LDQWLCEK (m/z 1034) with aronia procyanidin dimers and trimers with a 10:1 ratio.

Dimeric and trimeric procyanidins exhibited antioxidant activity towards selected peptides. Possible interaction products were not detected. For further investigations of interaction, higher concentration and ratio of peptides and procyanidins as well as expanding the MS detection range are recommended. Intensive research of oxidation products of ALA peptides is needed to accomplish a thorough understanding of the oxidation and other interaction reactions of ALA peptides with other food constituents.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>3-OH-Kyn</td>
<td>3-hydroxy-kynurenine</td>
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<tr>
<td>5-OH-Trp</td>
<td>5-hydroxy-tryptophan</td>
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<tr>
<td>A, Ala</td>
<td>Alanine</td>
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<tr>
<td>ACN</td>
<td>Acetonitrile</td>
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<td>ALA</td>
<td>Alpha-lactalbumin</td>
</tr>
<tr>
<td>BPC</td>
<td>Base peak chromatogram</td>
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<td>C, Cys</td>
<td>Cysteine</td>
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<td>D, Asp</td>
<td>Aspartate</td>
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<tr>
<td>DiOia</td>
<td>Dioxindolylalanine</td>
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<td>DNPH</td>
<td>2, 4-dinitrophenylhydrazones</td>
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<tr>
<td>DOPA</td>
<td>3,4-dihydroxyphenylalanine</td>
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<td>E, Glu</td>
<td>Glutamine</td>
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<tr>
<td>EIC</td>
<td>Extracted ion chromatogram</td>
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<td>F, Phe</td>
<td>Phenylalanine</td>
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<td>FL</td>
<td>Fluorescence</td>
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<td>G, Gly</td>
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<td>H, His</td>
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<td>HClO</td>
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<td>Isoleucine</td>
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<td>Kynurenine</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>MCO</td>
<td>Metal-catalyzed oxidation</td>
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<td>MeOH</td>
<td>Methanol</td>
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<td>NFK</td>
<td>N-formylkynurenine</td>
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<td>NP</td>
<td>Normal phase</td>
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<td>Oia</td>
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<td>P, Pro</td>
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<tr>
<td>PO·</td>
<td>Phenoxyl radical</td>
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<tr>
<td>PIC</td>
<td>3a-hydroxypyrroloindole-2-carboxylic acid</td>
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<td>R, Arg</td>
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<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
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<td>Peroxy radical</td>
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<td>RSSR⁻</td>
<td>Disulfide radical anion</td>
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<td>Serine</td>
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<tr>
<td>SPE</td>
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<td>Trifluoroacetic acid</td>
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<tr>
<td>T, Thr</td>
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<td>TIC</td>
<td>Total ion chromatogram</td>
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<td>Tra</td>
<td>Tryptamine</td>
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<td>UV-Vis</td>
<td>Ultraviolet-visible</td>
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<td>Tryptophan</td>
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<tr>
<td>Y, Tyr</td>
<td>Tyrosine</td>
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REFERENCES
1 INTRODUCTION

Alpha-lactalbumin (ALA) is an important whey protein in bovine and human milk, which plays a role in human metabolism. It helps to reduce stress (Markus et al. 2000), inhibits growth of cancer cells (Sternhagen and Allen 2001), protects against gastric mucosal injury (Matsumoto et al. 2001) and has antimicrobial activity (Pellegrini et al. 1999). ALA has a molecular mass of 14200 Da and involves 123 amino acids.

Oxidation is one of the key degradation pathways of proteins and it is involved in food storage, industrial biotechnology, aging and age-related diseases (Törnvall 2010). In foodstuffs, protein oxidation causes the deterioration of food quality and shortens their shelf life, due to the undesirable physical, chemical and functional changes in foods (Koivumäki et al. 2012). Proteins and free amino acids are prone to be oxidized by reactive oxygen species (ROS). In ALA, the amino acids susceptible to oxidation include methionine, cysteine, histidine, tyrosine and tryptophan (Törnvall 2010).

In the digestion process and food systems, proteins may react with other food constituents. The interactions between berry procyanidins and tryptic peptides of ALA may influence the antioxidant activity of procyanidins, depending on the temperature, pH, peptide concentration and the structure of procyanidins (Ozdal et al. 2013). Procyanidins can interact with peptides in reversible and irreversible ways, with non-covalent bonds and covalent bonds, respectively.

Procyanidins are the dominant phenolics in Aronia melanocarpa, which is also known as chokeberry. Procyanidins are phenolics that consist exclusively of epicatechin and catechin monomeric flavan-3-ols. They are polar compounds and prefer polar solvents to be extracted, for example, acetone and methanol. The most efficient extraction of high-molecular weight proanthocyanidins is obtained with acetone, methanol and water (2:2:1) containing 0.01% formic acid (Kalt et al. 2008, Hellström and Mattila 2008). Procyanidins are commonly analyzed with high performance liquid chromatography connected with UV-Vis, fluorescence, mass spectrometry or nuclear magnetic resonance detectors.

Procyanidins have received attention due to their role of astringency, color stability and bitterness in plant foods, but are more renowned for their antibacterial, antiviral, anticarcinogenic, anti-inflammatory and antioxidant activities. The antioxidant activities of procyanidins are performed by three mechanisms, regarding scavenging radical species.
such as reactive oxygen and nitrogen species (ROS/ RNS); chelating metals related to free radical production and protein binding  (Muralidhara and Prakash 1995, Cotelle 2001).

The aim of the thesis was to study the oxidation of tryptic peptides of ALA and their oxidation in the presence of procyanidins. The literature review part describes the ALA and its oxidation, aronia procyanidins and their chemistry and analysis, as well as protein and phenolic interactions. The experiment section involves the separation and analysis of dimeric and trimeric procyanidins, digestion and fractionation of ALA, oxidation of peptides with and without procyanidins, and LC-MS analysis of the oxidation products.
2 LITERATURE REVIEW

2.1 Alpha-lactalbumin

Alpha-lactalbumin (ALA) is an important whey protein in bovine and human milk. In human milk, ALA is the predominant whey protein, which has a 74% conserved amino acid sequence homology with bovine α-lactalbumin. While in bovine milk, ALA is the second major protein constituting 20-25% of the whey protein. Alpha-lactalbumin plays a role in human metabolism. It helps to reduce stress (Markus et al. 2000), inhibits growth of cancer cells (Sternhagen and Allen 2001), protects against gastric mucosal injury (Matsumoto et al. 2001) and has antimicrobial activity (Pellegrini et al. 1999).

ALA has a compact globular structure with a molecular mass of 14200 Da. It is a small acidic, single-chain Ca\(^+\) binding protein, involving 123 amino acids without free thiol groups (Stănciuc and Râpeanu 2010). The three dimensional structure of ALA (shown in Figure 1) involves α-helical and β-sheet domain, separated by a cleft (Chrysina et al. 2000).

![Figure 1. Structure of bovine holo α-lactalbumin (Chrysina et al. 2000).](image)

The α-helical domain consists of four α-helices and two 3\(_{10}\) helices while β-sheet domain contains a tripleβ-sheet, a 3\(_{10}\) helix and loops (Wijesinha-Bettoni et al. 2001).
2.1 Amino acid composition of ALA

ALA has two predominant genetic variants, A and B. They differ in the amino acid composition at position 10 of the protein, that is, A variant has Glu, while B variant contains Arg (Stănciuc and Râpeanu 2010). The B variant is often used as the reference protein, which is consisted of alanine (A, Ala), arginine (R, Arg), asparagine (N, Asn), aspartate (D, Asp), cysteine (C,Cys), glutamine (Q, Gln), glutamate (E, Glu), glycine (G, Gly), histidine (H, His), isoleucine (I, Ile), leucine (L, Leu), lysine (K, Lys), methionine (M, Met), phenylalanine (F, Phe), proline (P, Pro), serine (S, Ser), threonine (T, Thr), tryptophan (W, Trp), tyrosine (Y, Tyr) and valine (V, Val). ALA consists of 123 amino acid residues stabilized by four disulfide bonds, Cys6-Cys120, Cys28-Cys111, Cys61-Cys77, and Cys73-Cys91. The amino sequence is displayed in Figure 2.

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<td>Val-Ser-Leu-Pro-Glu-Trp-Val-Cys-Thr-Thr-Phe-His-Thr-Ser-Gly-Tyr-Arg-Thr-Gln-Ala-</td>
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<td>17</td>
<td>18</td>
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<td>20</td>
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<td>22</td>
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<td>Cys-Lys-Asp-Asp-Gln-Asn-Pro-His-Ser-Ser-Asn-Ile-Cys-Asn-Ile-Ser-Cys-Asp-Lys-Phen-</td>
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<tr>
<td>Ile-Asn-Tyr-Trp-Leu-Ala-His-Ile-Asp-Ala-Leu-Cys-Ser-Glu-Lys-Asp-Gln-Trp-Leu-Cys-</td>
<td>44</td>
<td>45</td>
<td>46</td>
<td>47</td>
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<td>50</td>
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<td>Glu-Lys-Leu-GH</td>
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</table>

Figure 2. Amino acid sequence of bovine α-Lactalbumin (Farrell et al. 2004)

ALA is a calcium binding metalloprotein, with calcium ion coordinated to β-carboxyl groups of three aspartic acid residues (Asp\(^{82}\), Asp\(^{87}\) and Asp\(^{88}\)), two carbonyl groups of the peptide backbone (Lys\(^{79}\) and Asp\(^{84}\)) and two water molecules (Chrysina et al. 2000).

2.1.2 Peptides of ALA formed during digestion

In terms of peptide analysis, enzymes are often used to hydrolyze protein, thus stimulating the gastric digestion. Typical enzymes include pepsin, trypsin, proteinase K, chymotrypsin and thermolysin (De Laureto et al. 1999, N’Negue et al. 2006). ALA is fairly resistant to digestion attributing to its globular structure or acid induced conformational change (N’Negue et al. 2006, Kamau et al. 2010). ALA is not prone to thermal denaturation and any treatment below 100°C allows the refolding of protein during cooling stage of enzyme hydrolysis (Kamau et al. 2010). Effect of different enzymes has been compared by Hernandez-Ledesma et al. (2005), involving pepsin, corolase PP and thermolysin. ALA is
hydrolyzed slowly and partially by trypsin but rapidly and totally by pepsin (Pintado and Malcata 2000). In Printado and Malcata's experiment (2000), ALA and β-lactoglobulin were incubated at 37°C for 0, 2.5, and 24h, digested by trypsin (pH 7.0) and pepsin (pH 2.0). Major hydrolysates of ALA were medium size peptides (2000-4000 Da) with trypsin, while pepsin resulted in small peptides (mainly < 500 Da), together with more free amino acids (Pintado and Malcata 2000). The main peptides gained by trypsin and pepsin are given in Tables 1 and 2.

**Table 1.** Peptides of ALA formed after digestion with trypsin (http://www.web.expasy.org).

<table>
<thead>
<tr>
<th>Mass</th>
<th>Position</th>
<th>MC</th>
<th>Peptide Sequence</th>
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<tbody>
<tr>
<td>4624.1361</td>
<td>17-58</td>
<td>0</td>
<td>GYGGVSLPEWVCTAFHTSGYDTQAIYQNDSTEYGLFQIN NK</td>
</tr>
<tr>
<td>1889.7752</td>
<td>63-79</td>
<td>0</td>
<td>DDQPHSSNIGNISCDK</td>
</tr>
<tr>
<td>1842.7338</td>
<td>80-93</td>
<td>0</td>
<td>FLDDDITDDIIMCVK</td>
</tr>
<tr>
<td>1200.6524</td>
<td>99-108</td>
<td>0</td>
<td>VGNYWLAHK</td>
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<tr>
<td>1034.4975</td>
<td>115-122</td>
<td>0</td>
<td>LDQWLCEF</td>
</tr>
<tr>
<td>653.3075</td>
<td>6-10</td>
<td>0</td>
<td>CEVFR</td>
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<tr>
<td>650.3176</td>
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<td>616.3457</td>
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<tr>
<td>549.2853</td>
<td>59-62</td>
<td>0</td>
<td>IWCK</td>
</tr>
</tbody>
</table>

1) ALA comes from Bos taurus (Bovine). 2) Digestion with trypsin with no missed cleavage. 3) All cysteines in reduced form. 4) Methionines have not been oxidized. 5) Displaying peptides with a mass bigger than 500 Dalton. 6) MC = missed cleavage

**Table 2.** Peptides of ALA formed after digestion with pepsin (http://web.expasy.org).

<table>
<thead>
<tr>
<th>Mass</th>
<th>Position</th>
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<th>Peptide Sequence</th>
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<td>INNKKW</td>
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<td>584.2412</td>
<td>32-36</td>
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</tr>
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</table>

1) ALA comes from Bos taurus (Bovine). 2) Digestion with pepsin (pH > 2) with no missed cleavage. 3) All cysteines in reduced form. 4) Methionines have not been oxidized. 5) Displaying peptides with a mass bigger than 500 Dalton. 6) MC = missed cleavage
2.2 Oxidation of ALA

2.2.1 Protein oxidation

Oxidation is one of the key degradation pathways of proteins and it is involved in food storage, industrial biotechnology, aging and age-related diseases (Törnvall 2010). In foodstuffs, protein oxidation causes the deterioration of food quality and shortens their shelf life, due to the undesirable physical, chemical and functional changes in foods (Koivumäki et al. 2012).

Proteins and free amino acids are prone to be oxidized by reactive oxygen species (ROS). Common ROS generating systems include pollutants in the atmosphere (ozone, N₂O₂, NO₂, cigarette smoke), exposure to X-, λ-, or UV-irradiation, by-products of normal metabolic process, such as autoxidation of reduced forms of electron carries, nitric oxide synthesis, oxidase catalyzed reactions, inflammatory reactions, nitric oxide synthesis, lipid peroxidation, and metal catalyzed reactions (Stadtman and Levine 2000). Hydroxyl radical (·OH) is one of the ROS, together with superoxide anion radical (O₂⁻), nitric oxide (NO⁻), peroxyl radicals (ROO⁻), peroxynitrite (ONOO⁻), singlet oxygen (¹O₂), hypochlorous acid (HOCl) and hydrogen peroxide (H₂O₂) (Swallow 1960, Garrison et al. 1962, Schuessler 1984, Garrison 1987).

Metal-catalyzed oxidation

Metal-catalyzed oxidation (MCO) system is the major source of protein oxidation damage since hydrogen peroxide and alkylperoxide used in MCO system are the most common end products of ROS generating system (Requena et al. 2003). By addition of metal, these relatively unreactive peroxides are converted to highly reactive hydroxyl radical or alkyl radical that can react with almost any organic substance (Stadtman and Levine 2000).

\[
\begin{align*}
\text{H}_2\text{O}_2 + \text{Cu}^+ \text{ or } \text{Fe}^{2+} & \rightarrow \text{Cu}^{2+} \text{ or } \text{Fe}^{3+} + \text{OH}^- + \text{HO}^- \quad (1) \\
\text{ROOH} + \text{Cu}^+ \text{ or } \text{Fe}^{2+} & \rightarrow \text{Cu}^{2+} \text{ or } \text{Fe}^{3+} + \text{OH}^- + \text{RO}^- \quad (2)
\end{align*}
\]

Metal-catalyzed protein oxidation is a site specific or "caged" process under physiological conditions, in which the ROS generated through MCO system prefer to react with amino acid residues at metal binding sites (Farber and Levine 1986, Chevion 1988, Stadtman 1990). The mechanism is illustrated by protein oxidation with a lysyl residue (Figure 3).
As displayed in Figure 3, Fe$^{3+}$ is first reduced to Fe$^{2+}$ (step a) and binds to the protein, forming coordination complex with the lysyl residue (step b). H$_2$O$_2$ is formed by the reduction of O$_2$ (step c) and then reacts with Fe$^{2+}$, generating OH-, HO∙ and a Fe$^{3+}$-protein complex (step d). The hydroxyl radical will abstract the hydrogen atom from the carbon atom of the lysyl residue, resulting in a carbon-centered radical (step e). The unpaired electron on this carbon-centered radical will be transferred to Fe$^{3+}$ in the complex, reducing it to Fe$^{2+}$ and the lysyl group is converted to an imino derivative (step f). At last, the imino derivative is hydrolyzed spontaneously, releasing NH$_3$, Fe$^{2+}$ and an aldehyde derivative of the lysyl residue (step g).

Oxidative damage of proteins

Oxidation of protein can cause modifications of amino acid side chains, further oxidation can lead to the cleavage of peptide bonds by α-amidation or diamide pathways, yielding α-keto-acyl or isocyanate derivatives (Stadtman and Levine 2003). Moreover, cross-linked protein aggregates can also form during oxidation (Törnvall 2010).

In terms of the oxidative modification of side chains, amino acids have different susceptibility to oxidation. Methionine and cysteine are most prone to oxidation due to the ease of oxidation of sulfur centers (Törnvall 2010). Histidine, tryptophan and tyrosine are also targets for oxidation (Li et al. 1995, Davies 2005). Some common side-chain
oxidation products of protein are given in Table 3.

Table 3. Common variants of amino acid side chain oxidation (Törnvall 2010).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Oxidized product</th>
<th>Mass shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine</td>
<td>Methionine sulfoxide</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Methionine sulfone</td>
<td>22</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cysteine sulfonic acid</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Cysteine (disulfide)</td>
<td>—</td>
</tr>
<tr>
<td>Cystine</td>
<td>Cleavage of disulfide bond and conversion to sulfonic acid</td>
<td>48$^a$</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Hydroxytryptophan</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>N-Formylkynurenine</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Kynurenine</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3-Hydroxykynurenine</td>
<td>16 + 4</td>
</tr>
<tr>
<td>Histidine</td>
<td>Dehydro-2-imidazolone</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Oxo-histidine</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>2-Imidazolone</td>
<td>16 + 1</td>
</tr>
<tr>
<td></td>
<td>5-Hydroxy-2-imidazolone</td>
<td>32 + 1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Dityrosine</td>
<td>(2M + H)$^+$ − 2</td>
</tr>
<tr>
<td></td>
<td>3-Chlorotyrosine$^d$</td>
<td>+35</td>
</tr>
<tr>
<td>Proline</td>
<td>Hydroxyproline/γ-glutamyl semialdehyde</td>
<td>+16</td>
</tr>
<tr>
<td></td>
<td>Pyroglutamic acid</td>
<td>+14</td>
</tr>
<tr>
<td>Arginine</td>
<td>γ-Glutamyl semialdehyde</td>
<td>−43</td>
</tr>
<tr>
<td>Lysine</td>
<td>Aminoadipic semialdehyde</td>
<td>−1</td>
</tr>
</tbody>
</table>

$^a$ +96 if both cysteines appear in the same peptide. $^b$ See Domingues et al.$^{15}$ for other products of tryptophan oxidation. $^c$ Only for oxidation by hypochlorous acid, which is generated in vivo by the phagocyte enzyme myeloperoxidase.$^{16}$

As shown in Table 3, protein oxidation can lead to the hydroxylation of aromatic and aliphatic amino acids with a mass increase of 16 amu, such as tryptophan and proline. Chlorination is also possible modification to amino acids, for instance, tyrosine oxidized with hypochlorous acid. Intra- and inter-disulfur bonds may form between cysteines. Carbonyl groups, such as ketones and aldehydes, can be generated by arginine, lysine, histidine and proline.

Markers of oxidative protein damage

Carbonyl compounds are widely considered as oxidation markers (Stadtman and Levine 2003). Carbonyl compounds are commonly measured with DNPH method by reaction with 2,4-dinitrophenylhydrazine converting to 2, 4-dinitrophenylhydrazones (DNPH) and measured with spectrophotometry (Oliver et al. 1987, Levine et al. 1994). Fluorescence detection can also be applied in analysis of carbonyls, whose excitation wavelength is 350 nm and emission wavelength between 400 to 500 nm regarding different carbonyl groups. Besides, tryptophan loss can be monitored as a sign of oxidation, which shows specific fluorescence at Ex 283 nm and Em 338 nm (Heinonen et al. 1998, Estevez et al. 2008). In
addition, formation of dityrosine can be another biomarker, with fluorescence excitation at 315 nm or 284 nm and emission wavelength of 400 nm (Malencik et al. 1996).

Recently, LC-MS method has been employed as a modern and sensitive technique to analyze protein oxidation products. Tryptophan side-chain oxidation derivatives can also be studied by LC-ESI-MS (Domingues et al. 2003, Szuchman-Sapir et al. 2008). Glutamic and aminoadipic semialdehydes have been analysed with LC-ESI-MS (Estevez et al. 2009). Koivumäki et al. (2012) have investigated oxidation of tryptic peptides of β-lactoglobulin and suggested that methionine containing peptides could be applied as oxidation markers.

2.2.2 Oxidative modifications of alpha-lactalbumin

In ALA, the amino acids susceptible to oxidation include methionine, cysteine, histidine, tyrosine and tryptophan (Törnvall 2010).

Oxidative modifications of cysteine and methionine

The ease of oxidation of sulfur centers makes cysteine and methionine the major targets for many oxidants. The mechanism of cysteine oxidation is quite complicated, yet not entirely understood (Xu and Chance 2007). According to the preferred targets of oxidants, the oxidation pathways can be divided into two categories, one-electron oxidation and two-electron oxidation (Winterbourn and Hampton 2008). Two-electron oxidants, such as hydrogen peroxide, will generate sulfenic acid (RSOH) as the primary oxidation product (Figure 4). This intermediate product undergoes secondary reaction and form intramolecular or intermolecular disulfides. The RSOH can also be further oxidized to sulfenic acid (RSO₂H) and sulfonic acid (RSO₃H) (Xu and Chance 2007). Even though hydrogen peroxide is a strong oxidant, the activation energy required is high and the reaction is slow. Cysteine is among the few amino acids that hydrogen peroxide can attack directly (Winterbourn and Hampton 2008).
Figure 4. Two-electron oxidation of cysteine with hydrogen peroxide (Xu and Chance 2007). The thiol group of cysteine can be oxidized to sulfenic acid (RSOH), sulfinic acid (RSO$_2$H) or sulfonic acid (RSO$_3$H). RSOH can also intramolecular or intermolecular disulfides (RSSR).

On the other hand, one-electron oxidation generates thyl radical (RS$^\cdot$) as the initial product. The thyl radical is formed through hydrogen abstraction from sulhydryl (RSH) by reactive oxygen species, such as hydroxyl radical. RS$^\cdot$ can further react with oxygen (O$_2$) or thilate (RS), generating a thyl peroxyl radical (RSOO$^\cdot$) or a disulfide radical anion (RSSR$^\cdot$), depending on the pH, oxygen and thyl concentrations (Xu and Chance 2007). RSOO$^\cdot$ can be transferred to sulfenic, sulfinic and sulfonic acid, disulfide and serine products. RSSR$^\cdot$ can react with molecular oxygen, resulting in molecular disulfide RSSR and superoxide O$_2^\cdot$ (Lal 1994).

Similar to cysteine, the sulfur-containing amino acid methionine is prone to be oxidized. Oxidation of methionine first yields methionine sulfoxide by addition of one oxygen atom (Figure 5). Further oxidation will lead to the formation of methionine sulphone (Rinalducci et al. 2008).

Figure 5. Oxidation of methionine. Methionine is first oxidized to methionine sulfoxide and further oxidized to methionine sulphone (Davies 2005).

Oxidative modifications of histidine

The oxidation products of histidine are complex and have not been characterized yet (Hawkins and Davies 2001). The major oxidation products include 2-oxohistidine, asparagine, and aspartic acid, which are displayed in Figure 6 (Xu and Chance 2007).
Figure 6. Major oxidation products of histidine. Histidine can be oxidized to 2-oxohistidine (+16 Da), aspartic acid (-22 Da) and asparagine (-23 Da) (Xu and Chance 2007).

The hydroxyl radical will attack the carbon at position 2, 4 or 5 of the imidazole ring first, resulting in the stable allyl-type radicals (Xu and Chance 2007). These radicals react with oxygen molecule and produce diverse oxidation products with different mass changes.

Oxidative modifications of tyrosine

In the presence of molecular oxygen, tyrosine can be oxidized to 3,4-dihydroxyphenylalanine (DOPA) by adding an oxygen to the carbon adjacent to the original hydroxyl group (Figure 7). When oxygen is absent, the yield of DOPA is lower and cross-linked derivatives can be formed, such as bityrosine (Xu and Chance 2007).

Figure 7. Oxidation products of tyrosine (Stadtman and Levine 2003). DOPA = 3,4 dihydroxyphenylalanine.

Oxidative modifications of tryptophan

The oxidation of tryptophan is complicated and irreversible (Rinalducci et al. 2008). The oxidation products can be 3a-hydroxypyrroloindole-2-carboxylic acid (PIC), β-oxindolylalanine (Oia), dioindolylalanine (DiOia), 5-hydroxy-tryptophan (5-OH-Trp), tryptamine (Tra), N-formylkynurenine (NFK), kynurenine (Kyn), and 3-hydroxykynurenine (3-OH-Kyn). The oxidation pathways are displayed in Figure 8.
Figure 8. Formation of tryptophan-derived oxidation products (Salminen and Heinonen 2008).

The oxidative attack appears on the pyrrole ring first (Simat and Steinhart 1998), resulting in diastereomer A and B of PIC, Oia (Kell and Steinhart 1990) and DiOia (Itakura et al. 1994). The following oxidation of the phenyl moiety will produce the 5-OH-Trp (Simat and Steinhart 1998). The decarboxylation of alanyl moiety can lead to the formation of Tra and indole derivatives. When the pyrrole ring of tryptophan breaks, NFK, Kyn, 3-OH-Kyn will be produced (Simat and Steinhart 1998).

2.3 Procyanidins and aronia berry

2.3.1 Occurrence of procyanidins in food

Berries are a good source of procyanidins, such as blueberries, blackberries, lingonberries, bog whortleberries, elderberries, and chokeberries (Gu et al. 2004, Määttä-Riihinen et al. 2004). Other commonly consumed food as grapes, kiwis, pears, apples, teas, skin of peanuts, the seed coat of almonds and certain cocoa are also rich in proanthocyanidins (Valls et al. 2009). The concentration of procyanidins with low degree of polymerization (DP) in nuts, cereal and legumes has been studied by Bittner et al. (2013). In apples (Malus silvestris), procyanidin of low molecular mass is the predominant proanthocyanidin. The pine (Pinus radiate) bark proanthocyanidin is exclusively
procyanidin of high mean DP (Huemmer and Schreier 2008). Procyanidins in cocoa vary in DP, ranging from monomers to polymers, which usually contain repeating subunits (Ortega et al. 2010). Proanthocyanidins still exist in processed food with varied and lowered concentration, such as red wine, grape juice, beer and chocolate (Valls et al. 2009).

2.3.2 Aronia procyanidins

Aronia berries originally grew in the eastern parts of North America and East Canada and migrated to Europe (Kulling and Rawel 2008). The name Aronia is the genus name for the common chokeberry, which has three species, of which two have been differentiated, regarding *Aronia melanocarpa* [Michx.] Elliot (black chokeberry, Aronia noir) and *Aronia arbutifolia* [L.] Elliot (red chokeberry, Aronia rouge) (Hardin 1973). The third, however, is considered as a hybrid.

The dominant phenolics in *Aronia melanocarpa* are polymeric proanthocyanidins, which are exclusively B type procyanidins (Oszmianski and Wojdylo 2005). The flavan-3-ol monomers constituting procyanidins are principally (-)epicatechin. The second predominant phenolics are anthocyanins that represent 25% of total phenolics. The anthocyanins are composed of four cyanidine glycosides, including 3-galactoside, 3-glucoside, 3-arabinoside and 3-xyloside (Oszmianski and Sapis 1988). Chlorogenic and neochlorogenic acids are also present, but in relatively low amount, constituting 7.5% of the total phenolics (Oszmianski and Wojdylo 2005). The proanthocyanidins in aronia are mainly polymers (DP> 10), constituting 81.72% of the total proanthocyanidins. Other components are monomers (0.78%), dimers (1.88%), trimers (1.55%), 4-6-mers (6.07%), 7-10-mers (7.96%) (Oszmianski and Wojdylo 2005).

The health benefits of Aronia berries have received great interest, regarding inhibition of cancer cell proliferation, antioxidant effects, antimutagenic activity, hepatoprotective effects, cardioprotective effects, and antidiabetes effects (Kulling and Rawel 2008). These effects are related to the presence of procyanidins, chlorogenic acids and anthocyanins.
2.4 Composition and chemistry of procyanidin

2.4.1 Structure of procyanidins

Procyanidins constitute the largest class of proanthocyanidins, which are composed of different flavan-3-ol monomers. Procyanidins consist exclusively of epicatechin and catechin monomeric flavan-3-ols. Procyanidins are primarily linked through B-type bonds, in which C4→C8 linkage (carbon-carbon bonds from the 4 position of one subunit to the 8 position of another) is more than C4→C6 linkage (Figure 9). A-type procyanidins occasionally occurs, containing two interflavan linkages, one C4→C8 or C4→C6 linkage and an additional ether bond between C2→O7 or C2→O5 (White et al. 2010).

![Figure 9. Examples of A- and B-type procyanidins](Huemmer and Schreier 2008)

The chemical structures of procyanidins are subject to pH. The oligomeric procyanidins undergo decomposition and isomerization at pH 2 (Zhu et al. 2002). The monomers, (2R, 3R)-epicatechin and (2R, 3S)-catechin, are stable in aqueous solution up to pH 5, but isomerize with increasing pH. Monomers and dimers immediately degenerated when the pH is above 9, resulting in brown color products (Huemmer and Schreier 2008). Moreover, the linkage type also makes a difference in the stability. The 4 β-8 bond is more stable than the 4 β-6 linkage. Kondo et al. (2000) have found that under oxidizing conditions, procyanidin configuration transformation occurred, from B-type to A-type. Procyanidins are quite vulnerable to oxygen, light, acid and alkaline. Freeze-drying has been considered an appropriate method for procyanidin storage (Matthews et al. 1997).

2.4.2 Bioactivities of procyanidins

Procyanidins received attention due to their role in astringency, color stability, and bitterness in plant foods (Kelm et al. 2006). However, the potential health effects of procyanidins have drawn more attention. Procyanidins are considered to have antioxidant,
antibacterial, antiviral, anticarcinogenic, anti-inflammatory, and vasodilatory activities (De Bruyne et al. 1999, Carnescchi et al. 2002, Cortes et al. 2002, Jayaprakasha et al. 2003, Garbacki 2004, Cheng et al. 2005). Lingonberry proanthocyanidins were reported to be powerful antioxidants in the oxidation of lactalbumin (Viljanen et al. 2004). Bog-whortleberry catechins, even in low concentration, demonstrated strong ability in inhibiting oxidation of human low-density lipoprotein (Heinonen 2007). In addition, the interflavan linkage type and the composition of procyanidins will also influence their biological effects. For instance, procyanidins bearing exclusively A-type are assumed to contribute to the prevention of type 2 diabetes mellitus or of urinary tract infections (Foo et al. 2000, Anderson et al. 2004). Moreover, procyanidin polymers exhibit different metabolic pathways with oligomers, which are assumed to be absorbed in the small intestine (Deprez et al. 2001, Gonthier et al. 2003, Rzeppa et al. 2012).

**Antioxidant activity of procyanidins**

The antioxidant activities of procyanidins are performed by three mechanisms, regarding scavenging radical species, such as reactive oxygen and nitrogen species (ROS/ RNS), chelating metals related to free radical production and protein binding (Muralidhara and Prakash 1995, Cotelle 2001).

The radical scavenging activities of procyanidins are attributed to their role of free radical acceptors and chain breakers in the oxidation process. Procyanidins provide a hydrogen atom to radicals (R) formed from protein oxidation or other molecules, resulting in phenoxy radical (PO•) (Equation 3). This phenoxy radical intermediate will not cause further chain reaction since it is comparatively stable. Furthermore, the phenoxy radical intermediates can react with other radicals and finally terminate the propagation (Equation 4) (Dai and Mumper 2010).

\[ R + POH \rightarrow RH + PO\cdot \quad (3) \]

\[ PO\cdot + R \rightarrow POR \quad (4) \]

The structure of the procyanidins contributes to their radical scavenging activities, regarding the aromatic ring and hydroxyl groups. The hydroxyl groups act as hydrogen or electron donators and the aromatic ring is able to delocalize an unpaired electron (Heim et al. 2002).
More specifically, the ortho-dihydroxyl groups on the B ring is the best electron donators, which generate highly stable radicals and participate in electron delocalization. The 3,5 hydroxyl groups in A and C rings contribute to the maximum radical scavenging potential, in which 3-hydroxyl group is important for antioxidant activity (Shahidi et al. 1992, Bors and Michel 2002).

Alternate to radical scavenging activity, the dihydroxyl groups of procyanidins are able to chelate metals to hinder metal-induced free radical formation (Dai and Mumper 2010). The procyanidins can chelate redox active metal ions, such as Cu\(^+\) or Fe\(^{2+}\), which originally react with hydrogen peroxide (H\(_2\)O\(_2\)) in Fenton reaction and produce highly reactive hydroxyl radicals (•OH) (Equation 1). Metal ions can conjugate procyanidins in different places, such as 3’, 4’-o-diphenol groups in the B ring, 3, 4 or 3, 5-o-diphenolic groups in the C ring (Figure 10) (Rice-Evans et al. 1997).

![Figure 10. Binding of metal ions and phenolics (Scalbert et al. 2005).](image)

Moreover, longer chain proanthocyanidins exhibit better antioxidant activities than shorter ones, which are proved by studies on the relationship between chain length and radical scavenging activity of procyanidins, regarding cocoa, almonds and grape seeds (Muselik et al. 2007, Spranger et al. 2008, Calderon et al. 2009). Similarly, in low-density lipoprotein (LDL), procyanidins with higher DP are better antioxidant (da Silva Porto et al. 2003). However, Steinberg et al. (2002) claimed that procyanidin chain length did not determine the ability to protect LDL from oxidation when monomer units were controlled. In addition, Viljanen et al. (2004) have compared the antioxidant activity of monomeric, dimeric and trimeric procyanidins and found procyanidin dimers and trimers were more efficient in liposome model. Contradictory to this, Määttä-Riihinen et al. (2005) did not observe different scavenging activities between monomeric, dimeric and trimeric procyanidins in methyl linoleate emulsion. In addition to DP, the interflavan linkage type (C4→C6 or C4→C8) will also influence the diverse antioxidant activity of procyanidins (da Silva Porto et al. 2003).
Prooxidant activity of procyanidins

Procyanidins can be converted to prooxidants by reacting with oxygen, producing quinones (P=O) and superoxide anion (O$_2^-$) as displayed in Equation 5 (Cotelle 2001). Moreover, the metal ions can promote the prooxidants activity of procyanidins as well by the following reactions (Equations 6-10)

$$\text{PO} \cdot + \text{O}_2 \rightarrow \text{P}=\text{O} + \text{O}_2^\cdot$$  \hspace{1cm} (5)

$$\text{Cu}^{2+} \text{ or } \text{Fe}^{3+} + \text{POH} \rightarrow \text{Cu}^+ \text{ or } \text{Fe}^{2+} + \text{PO} \cdot + \text{H}^+$$  \hspace{1cm} (6)

$$\text{PO} \cdot + \text{RH} \rightarrow \text{POH} + \text{R}.$$  \hspace{1cm} (7)

$$\text{R} \cdot + \text{O}_2 \rightarrow \text{ROO}.$$  \hspace{1cm} (8)

$$\text{ROO} \cdot + \text{RH} \rightarrow \text{ROOH} + \text{R}.$$  \hspace{1cm} (9)

$$\text{ROOH} + \text{Cu}^+ \text{ or } \text{Fe}^{2+} \rightarrow \text{Cu}^{2+} \text{ or } \text{Fe}^{3+} + \text{RO} \cdot + \text{OH}^-$$  \hspace{1cm} (10)

Under the condition that promotes the autoxidation of procyanidins, such as at high pH, high concentrations of transition metal ions and oxygen present, these antioxidants can act as prooxidants. For example, Salminen et al (2008 a, b) have discovered the promotion of tryptophan loss during oxidation by procyanidin B1 and B2 and blamed the inappropriate concentration. It was explained as that interaction between peptide and phenolics was closely associated with the antioxidant or prooxidant activity of phenolics and therefore antioxidants might turn to prooxidants when they lay beyond optimum (Van Acker et al. 1996). Compared with small phenolics, which are more prone to oxidation, high molecular weight tannins exhibit little or no prooxidants activity (Hagerman et al. 1998).

2.5 Analysis of proanthocyanidins

2.5.1 Extraction of proanthocyanidins

Proanthocyanidins are polar compounds and prefer polar solvents to be extracted, for example, acetone and methanol. Ethyl acetate can be used to extract procyanidin monomers and low-molecular–weight compounds (Määttä-Riihinen et al. 2004). Low-molecular-weight procyanidins can also be extracted with acid methanol (Koga et al. 1999, Sano et al. 2003). The most efficient extraction of high-molecular weight proanthocyanidins is obtained with acetone, methanol and water (2:2:1) containing 0.01%
formic acid (Kalt et al. 2008, Hellstrom and Mattila 2008). However, the proanthocyanidins cannot be extracted completely, especially prodelphinidins, which are much more unextractable than procyanidins (Huemmer and Schreier 2008). For additional separation, polyamide, Sephadex LH-20 and cartridges can be employed (Shoji et al. 2006, Fan et al. 2007).

2.5.2 Analysis of berry procyanidins by high performance liquid chromatography

High performance liquid chromatography is the most frequently used analytical technique in separation and identification of phenolics (Carrasco-Pancorbo et al. 2007, Ruberto et al. 2007). Proanthocyanidins have been analyzed with HPLC in many food samples. Table 4 summarizes the extraction and HPLC methods for proanthocyanidin analysis in berries. Both normal and reversed phase HPLC can be used to analyze procyanidins, for example, Figure 11 shows the HPLC chromatogram of proanthocyanidins isolated from Chinese hawthorn fruit stone with RP-HPLC and NP-HPLC.

![Figure 11](image.png)

**Figure 11.** (A) Reversed-phase HPLC chromatogram of Chinese hawthorn fruit stone proanthocyanidins after thiolytic degradation. Peaks are as follows: 1, catechin; 2, epicatechin 3, epicatechin gallate 4, A-type dimer 5, epigallocatechin benzylthioether; 6, A-type trimer benzylthioether; 7, catechin; 8, epicatechin benzylthioether; 9, A-type dimer benzylthioether; and 10, benzylmercaptan. (B) Normal-phase HPLC-ESI-MS chromatogram of Chinese hawthorn fruit stone proanthocyanidins. Peaks are as follows: 1, epicatechin. 2, catechin; 2a, A-type dimer; 3a, A-type trimer; 4a, A-type tetramer and 5a, A-type pentamer, respectively (Chai 2013).
<table>
<thead>
<tr>
<th>Source</th>
<th>Extraction</th>
<th>HPLC</th>
<th>Solvent</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lowbush blueberry and selected food samples</td>
<td>acetone/water/HAc (70:29.5:0.5) Sephadex LH-20</td>
<td>NP</td>
<td>A: CH₂Cl₂</td>
<td>(Gu et al. 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C: 50% HAc</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: MeOH</td>
<td></td>
</tr>
<tr>
<td>Aronia melanocarpa berry</td>
<td>acetone/water/HAc (70:29.5:0.5)</td>
<td>NP</td>
<td>A: CH₂Cl₂</td>
<td>(Wu et al. 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C: 50% HAc</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: MeOH</td>
<td></td>
</tr>
<tr>
<td>Saskatoon berry</td>
<td>MeOH/water (80:20)</td>
<td>NP</td>
<td>A: CH₂Cl₂</td>
<td>(Hellström et al. 2007)</td>
</tr>
<tr>
<td></td>
<td>Acetone/water (80:20)</td>
<td></td>
<td>C: 50% HAc</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sephadex LH-20</td>
<td></td>
<td>B: MeOH</td>
<td></td>
</tr>
<tr>
<td>Bilberry, lowbush blueberry, highbush blueberry, rabbit eye blueberry,</td>
<td>acetone/MeOH/HAc (23:77:10) Sephadex LH-20</td>
<td>NP</td>
<td>A: MeOH</td>
<td>(Kalt et al. 2007)</td>
</tr>
<tr>
<td>lingonberry, strawberry</td>
<td></td>
<td></td>
<td>C: 50% HAc</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: CH₂Cl₂/MeOH/water/HAc (41:7:1:1)</td>
<td></td>
</tr>
<tr>
<td>Bilberry, lowbush blueberry, highbush blueberry, rabbit eye blueberry,</td>
<td>acetone/MeOH/water/HAc (40:40:20:0.01), Sep C18 Sephadex LH-20</td>
<td>NP</td>
<td>A: MeOH</td>
<td>(Kalt et al. 2008)</td>
</tr>
<tr>
<td>lingonberry, strawberry</td>
<td></td>
<td></td>
<td>C: 50% HAc</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: CH₂Cl₂</td>
<td></td>
</tr>
<tr>
<td>Cranberry pomace</td>
<td>acetone/water/HAc (70:29.5:0.5) Sephadex LH-20</td>
<td>NP</td>
<td>A: ACN/HAc (98:2)</td>
<td>(White et al. 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: MeOH/water/HAc (95:3:2)</td>
<td></td>
</tr>
<tr>
<td>Strawberry</td>
<td>acetone/water/HAc (70:29.5:0.5) Sep C18 Sephadex LH-20</td>
<td>NP</td>
<td>A: ACN/HAc (98:2)</td>
<td>(Buendia et al. 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: MeOH/water/HAc (95:3:2)</td>
<td></td>
</tr>
<tr>
<td>Fruit stone of Chinese hawthorn</td>
<td>acetone/water (70:30) Sephadex LH-20</td>
<td>NP</td>
<td>A: CH₂Cl₂</td>
<td>(Chai 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C: 50% HAc</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: MeOH/water/HAc (41:7:1:1)</td>
<td></td>
</tr>
<tr>
<td>Blueberry</td>
<td>acetone/water/HAc (70:29.5:0.5) Sephadex LH-20</td>
<td>NP</td>
<td>A: ACN/HAc (99:1)</td>
<td>(Howard et al. 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: MeOH/water/HAc (95:4:1)</td>
<td></td>
</tr>
<tr>
<td>Vaccinium berry</td>
<td>EtAc</td>
<td>RP</td>
<td>A: water/formic acid (95:5)</td>
<td>(Määttä-Riihinen et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>Sephadex LH-20</td>
<td></td>
<td>B: ACN</td>
<td></td>
</tr>
<tr>
<td>Manitoba berry, chokeberry, seabuckthorn</td>
<td>acetone/water/HAc (70:29.5:0.5) Sephadex LH-20</td>
<td>RP</td>
<td>A: water/formic acid (99.9:0.1)</td>
<td>(Hosseinian et al. 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: MeOH</td>
<td></td>
</tr>
<tr>
<td>Lingenberry and cranberry</td>
<td>acetone/water (70:30) Sephadex LH-20</td>
<td>RP</td>
<td>A: water/formic acid (99.5:0.5)</td>
<td>(Kylli et al. 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: ACN</td>
<td></td>
</tr>
<tr>
<td>Fruit stone of Chinese hawthorn</td>
<td>acetone/water (70:30) Sephadex LH-20</td>
<td>RP</td>
<td>A: water/TFA (99.5:0.5)</td>
<td>(Chai 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: ACN</td>
<td></td>
</tr>
<tr>
<td>Bark of Aronia melanocarpa</td>
<td>acetone/water (70:30), EtAc Sephadex LH-20</td>
<td>RP</td>
<td>A: water/TFA (99.95:0.05)</td>
<td>(Braunlich et al. 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: ACN/TFA (99.95:0.05)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: NP, normal phase; RP, reversed phase; HAc, acetic acid; EtAc, ethyl acetate; MeOH, methanol; ACN, acetonitrile; TFA, trifluoroacetic acid; SPE, solid phase extraction.
Reversed-phase liquid chromatography separation

RP-HPLC with non-polar C18 or C8 column has been the primary technique to analysis proanthocyanidins, which allows the separation of procyanidins with the same DP (Valls et al. 2009). As can be seen from Figure 11A, procyanidin monomers, catechin and epicatechin elute in separate peaks (peak 1 and peak 2). Generally, 0.1-5% acids, such as acetic acid, formic acid and trifluoroacetic acid are added to the solvent to improve the affinity to non-polar column. However, the resolution of RP-HPLC is limited up to proanthocyanidin tetramers, as can be seen from Figure 11A. It is because the number of isomers increases with increasing DP, proanthocyanidin oligomers and polymers usually result in unresolved hump or drift of the baseline (Kallithraka et al. 1995).

Normal-phase liquid chromatography separation

Normal-phase HPLC, which is commonly equipped with silica or diol columns, separates proanthocyanidins according to their DP. Figure 11B clearly shows that proanthocyanidins elute with increasing DP. With silica column, NP-HPLC can separate proanthocyanidins efficiently up to decamers in apple, pine bark power, lingonberry, grape, cocoa and fruit stone of Chinese hawthorn (Counet et al. 2004, Hellström and Mattila 2008, Chai et al. 2014). The commonly utilized gradient of dichloromethane and methanol in acetic acid is not capable of eluting polymeric proanthocyanidin with DP>10, which eluted as a single peak at the end of the chromatogram (Gu et al. 2002). However, the chlorinated solvents in mobile phase may cause environmental related problems. An alternate gradient of acetone and hexane can separate procyanidins up to pentamer, yet still leave higher DP oligomers absorbed in the silica phase (Shoji et al. 2006). Therefore, Kelm et al (2006) adopted a diol column and a slight acidified gradient of acetonitrile and methanol, which received better separation than silica column (Kelm et al. 2006).

Detection of proanthocyanidins

HPLC is often connected with UV-Vis, fluorescence, mass spectrometry or nuclear magnetic resonance detectors to improve identification and characterization of procyanidins. Proanthocyanidins have two characteristic maxima in UV-Vis spectra, one at 200-220 nm and the other at 278 nm (Scharbert et al. 2004). Moreover, homogeneous procyanidin exhibits a symmetric maximum spectrum at 280 nm, while the spectrum becomes asymmetric when prodelphinidin is mixed with procyanidin (Rohr et al. 2000). Compared with UV-Vis detector, fluorescence detector is more effective and sensitive,
which is set at 276 nm for excitation and 316 nm for emission (Huemmer and Schreier 2008). The hyphenation of HPLC to mass spectrometers like TOF, qTOF and QIT provides a potent tool for structural identification. Before entering the mass spectrometer, ionization of procyanidins need to be performed preferably in negative mode, due to the acidity of phenolic protons (Hammerstone et al. 1999, Es-Safi et al. 2006, Hellström et al. 2007, Rzeppa et al. 2012). However, the acid in the mobile phase which is used to enhance efficiency in the negative mode, prefers to form acid/phenolate adduct, resulting in more complex spectra interpretation (Gonzalez-Manzano et al. 2006). Therefore, in many experiments, positive mode is also applied (Määttä-Riihinen et al. 2005, Gonzalez-Manzano et al. 2006, Kylli et al. 2011, Braunlich et al. 2013).

2.6 Protein and phenolic interactions

Interactions between proteins and phenolics can be reversible or irreversible, leading to changes in both compounds. The antioxidant activities of phenolics, as well as the in-vitro and in-vivo bioavailability may be changed (Le Bourvellec and Renard 2012). Many factors can influence the interactions, such as temperature, pH, type of protein, protein concentration, and type and structure of phenolics (Ozdal et al. 2013).

2.6.1 Reversible interactions

Reversible interactions have been studied thoroughly, which involve non-covalent forces, such as hydrogen binding, hydrophobic binding and van der Waal forces (Charlton et al. 2002, Jobstl et al. 2004, Poncet-Legrand et al. 2006, Richard et al. 2006). Van der Waals interactions are the weakest intermolecular attractions between molecules, which are driven by induced electrical interactions. Hydrogen bonding is a weak association formed between an electronegative acceptor atom and a hydrogen atom that is originally bonded to an electronegative donor atom, such as N, O or S (Lodish H, Berk A, Zipursky SL, et al. 2002). Compared with hydrogen bonding and Van der Waals interactions, hydrophobic interactions are stronger among nonpolar groups (Damodaran 1996). Hydrophobic interaction is the tendency of nonpolar compounds to accumulate in aqueous solutions (Chandler 2005). The mechanism of non-covalent interactions is displayed in Figure 12.
Hydrophobic interactions are the main driving force towards the aggregation of proteins and phenolics and could be strengthened by hydrogen binding (Haslam 1996). As revealed in Figure 12, hydrophobic interactions occur between aromatic rings of phenolics and the hydrophobic sites of proteins, such as the pyrrolidine rings of proline (Charlton et al. 2002, Richard et al. 2005, Poncet-Legrand et al. 2006). Hydrogen binding appears between hydroxyl groups of phenolics and the hydrogen acceptor sites of proteins (Jobstl et al. 2004, Richard et al. 2006). The ionic binding is weak compared with other interactions and could happen between the positively charged amino group of lysine and the hydroxyl group of phenolics (Le Bourvellec and Renard 2012).

2.6.2 Irreversible interactions

In irreversible interactions, proteins and phenolics are covalently connected. The covalent interaction involves the oxidation of phenolics, the formation of o-quinones or o-semiquinones and carbocations from phenolics (Le Bourvellec and Renard 2012). The reactions between proteins and phenolics are illustrated in Figure 13.
As shown in Figure 13, the diphenol groups of phenolics (1) are first oxidized to an orthoquinone by molecular oxygen (Damodaran 1996), which then dimerize (2) or react covalently with amino or sulfhydryl side chains of proteins, regenerating hydroquinone (Strauss and Gibson 2004). The hydroquinone can be oxidized again and connect to another protein, forming a cross-link (3) (Arts et al. 2001). The cross-link can also be produced by dimerization of two quinones (Arts et al. 2002).
3 EXPERIMENTAL RESEARCH

3.1 Aims of the study

The aim of the thesis was to study the oxidation of tryptic peptides of ALA and their oxidation in the presence of procyanidins.

3.2 Materials and methods

3.2.1 Materials

The aronia juice proanthocyanidins (aronia 1, 2, 3), which were separated with Sephadex LH-20 open column from Aronia melanocarpa juice by Koivumäki, Zeng and Liu, was used to further isolate procyanidin dimers and trimers. Procyanidin B2 was a product of Extrasynchthese (GENAY Cedex). Chromatographically purified and lyophilized α-lactalbumin (ALA) from bovine milk, was obtained from Sigma Aldrich, Inc. (St. Luois, MO, USA). Sequencing grade modified trypsin was from Promega Corp./BioFellows (Madison, WI, USA). The regents used in the analysis including ammonium bicarbonate, hydrogen peroxide (30% wt solution in water) and iron (III) chloride (regent grade, 97%) were obtained from Sigma Aldrich (Steinheim, Germany). The L-(+)-ascorbic acid came from Sigma Aldrich, Inc. (China) and PIPES-buffer [piperazine-1, 4-bis (2-ethanesulfonic acid)] was from Fluka BioChemika (Buchs, Switzerland). HPLC grade acetonitrile, methanol and dichloromethane, reagent grade formic acid (98-100%) were supplied by Sigma Aldrich (Steinheim, Germany). The Milli-Q water used was purified by the Milli-Q system (Millipore Corp., Bedford, MA, USA).

3.2.2 Methods

The outline of the methods is displayed in Figure 14.
Separation of dimeric and trimeric procyanidins from aronia juice proanthocyanidins (Preparative-HPLC)

Identification and quantification of dimeric and trimeric procyanidins (UHPLC, NP-HPLC)

In-liquid digestion of α-lactalbumin
(Trypsin, 37°C, 50 mM NH₄HCO₃ buffer, overnight)

Fractionation of tryptic peptides
(Preparative-HPLC)

Concentration measurement
(Spectrophotometer)

Preparation of samples
(2 sets of triplicate samples)

Peptide m/z 1034 +Dimeric and trimeric procyanidins (73:7.3 μg/mL)

Peptide m/z 936 +Procyanidin B2 standard (320:32 μg/mL)

Concentrated dimeric and trimeric procyanidins

Peptide m/z 1034

Peptide m/z 936

Oxidation (40 °C)
\( \text{H}_2\text{O}_2, \text{FeCl}_3, \text{ and ascorbic acid in PIPES} \)

Analysis with LC-MS

Procyanidin B2 standard

Figure 14. The outline of the methods.

Separation of procyanidin dimers and trimers

Procyanidin dimers and trimers were fractionated and collected from aronia juice proanthocyanidins with preparative-HPLC. The preparative-HPLC which involved a Waters 2767 sample manager, Waters 2545 binary gradient module and a Waters system fluidic organizer was fitted with a 250x20 mm i.d. 5 μm YMC-Pack diol-120-NP column,
coupled with Waters 2996 PDA and Waters 2475 fluorescence detector. The method was based on Hellström et al (2007). The mobile phases were: A, dichloromethane/methanol/MQ-water (42:7:1, v/v/v) and B, dichloromethane/methanol/MQ-water (5:44:1, v/v/v). The gradient program started with 100% A, followed by 0-30% B, 0-45 min; 30% B, 45-65 min; 30-85% B, 65-66 min; 85% B, 66-70 min; 85-30% B, 70-71 min; 30-0% B, 71-73 min; 0% B, 73-80 min. The injection volume was 4 mL and the flow rate was 15 mL/min. The separation was monitored using both UV (λ = 280 nm) and fluorescence detection (Ex. 280 nm & Em. 324 nm). Fractions were collected every one minute.

Identification and quantification of procyanidin dimers and trimers

All the fractions obtained from preparative-HPLC were analyzed on UHPLC with the method of Kylli et al. (2011) to check the purity of procyanidin dimers and trimers. The pure fractions from the preparative-HPLC were combined and quantified with the NP-HPLC. The NP-HPLC was equipped with a Waters717 plus autosampler, a Waters 510 module and a 515 HPLC pump. It was coupled with Waters 996 PDA and Waters 2475 fluorescence detector. The column was Silica Luna column (Phenomenex), 250×4.6 mm, i.d. 5 µm, with the column temperature set at 35 °C. The mobile phases were as follows: A, dichloromethane/methanol/MQ-water/acetic acid (41:7:1:1, v/v/v/v) and B, dichloromethane/methanol/MQ-water/acetic acid (5:43:1:1, v/v/v/v). The separation was started with 100% A, followed by 0-13.5% B, 0-20 min; 13.5-29.2% B, 20-50 min; 29.2-100% B, 50-55 min; 100% B, 55-60 min. The injection volume was 20 µL with the flow rate 1 mL/min. The separation was monitored using both UV (λ = 280 nm) and fluorescence detection (Ex. 280 nm & Em. 323 nm).

The concentration of the combined fractions was determined by procyanidin B2 standard curve made by Kahila (2014). The standard curve equation was y = 29787616.81x - 2046943.11, where y represented the peak area and x stood for the concentration. The dimeric and trimeric procyanidins were added to peptides LDQWLCEK (m/z 1034) later, with a ratio of 1:10, which were then subjected to oxidation. Separate injections of concentrated dimeric and trimeric procyanidins (492 µg/mL) were performed with LC-MS (LC-MS methods are described in the last chapter).
In liquid digestion of α-lactalbumin (ALA) with modified trypsin

ALA was cleaved by modified trypsin into peptides that were then used for oxidation and interaction with procyanidins dimers and trimers. The digestion was performed in Eppendorf tubes at 37°C overnight. The amount of ALA weighed into each Eppendorf tube was 10 mg, with 1 mL of 50 mM ammonium bicarbonate (NH₄HCO₃, pH 8) as the dissolving buffer. 40 µg of trypsin, which dissolved in its own resuspension buffer, was added to the final solution in the ratio of 1:250 (w/w). The sample was incubated in the oven after sufficient stirring and stopped by freezing at -20 ºC overnight. In order to collect enough peptides for the later oxidation study, 17 digestions were performed.

Fractionation of ALA with Preparative-HPLC

Enzymatically digested ALA was fractionated with preparative HPLC (Waters Corp., Milford, MA, USA), which consisted of Waters 2767 sample manager, Waters 2545 binary gradient module mode, an active flow splitter (1:100), Waters system fluidic organizer and was coupled with a Waters 2996 PDA detector and a single quadrupole (ZQ) mass spectrometer. The separation was performed with a Waters XBridge™ Prep BEH130, C18.5 µm, 10×250 mm column and analysed using MassLynx 4.1 software (Waters) with a modified method from (Koivumäki et al. 2012). The mobile phase consisted of gradient performed with MQ-water and acetonitrile, both of which contained 0.1% (v/v) formic acid. A volume of 950 µL of peptide sample was injected each time and the flow rate was 3.5 mL/min throughout the run. The gradient started with 5% acetonitrile and kept constant for 2 minutes. Then the content of acetonitrile rose linearly to 10% within 45 min. The following washing step caused a fast raise of acetonitrile to 80% for 4 min to rinse the column. Then acetonitrile dropped back to 5% within 1 min and lasted 7 min to finish the whole run. Desired peptides were collected by the fraction collector based on the programmed m/z value list. Fractions of two different peptides (Table 5) were chosen and stored in plastic falcon tubes at -20 ºC for further analysis.

Table 5. The tryptic ALA peptides fractionated and collected by preparative-HPLC

<table>
<thead>
<tr>
<th>Peptide fractionated</th>
<th>[M+H]⁺</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>WLAHKALC</td>
<td>m/z 936</td>
<td>Trp-Leu-Ala-His-Lys-Ala-Leu-Cys</td>
</tr>
<tr>
<td>LDQWLCEK</td>
<td>m/z 1034</td>
<td>Leu-Asp-Gln-Trp-Leu-Cys-Glu-Lys</td>
</tr>
</tbody>
</table>
Fractions that contain respectively peptides m/z 936 and m/z 1034 were concentrated by combination and evaporation of the solvent with a rotary evaporator (Rotavapor-R, Büchi Labortechnik AG, Flawil, Switzerland), using a water bath at 30 °C. During the combination, the falcon tubes were rinsed with 100% acetonitrile at least twice. The combined samples were stored in pure acetonitrile at -20 °C.

**Oxidation of peptides with and without procyanidins**

Prior to the oxidation of each concentrated peptides, the concentration was measured. The measurement was performed on a ND-1000 spectrophotometer (Nano Drop, Fisher Scientific, USA) under the program of Protein A-280 at wavelength of 280 nm. The general reference was applied, which was based on the absorbance of a 1 mg/mL protein solution at 280 nm when the path length is 1 cm.

The oxidation was conducted similarly to Koivumäki (2012). The samples were then evaporated to dryness with nitrogen, dissolved into 15 mL of freshly made 15 mM PIPES-buffer solution (pH 6.0), containing 0.1 mM ascorbic acid and 10 μM FeCl₃.

The solution was then divided into two groups, peptide group without procyanidins and peptide/procyanidin group in triplicates. Procyanidin dimers and trimers solution (7.3 μg/mL) was added to peptide LDQWLCEK m/z 1034 (73 μg/mL), while procyanidin B2 standard (32μg/mL) was added to peptide WLAHKALC m/z 936 (320 μg/mL) to make a 1:10 procyanidin to peptide ratio. The oxidation was initialled by addition of H₂O₂, with a concentration of 1 mM.

The samples were oxidized in an oven of 40 °C with magnet stirring operating. The oxidation experiment lasted for 14 days and subsampling was carried out on days 0, 1, 7 and 14. The samples were stirred adequately before subsamples of 200 μL each drawn into Eppendorf tubes. All subsamples collected were stored at -20 °C before analysis.

**LC-MS analysis of peptide-phenolic interactions**

All subsamples as well as the procyanidin B2 standard (886 μg/mL), procyanidin dimers and trimers (492 μg/mL) were characterized with Agilent 1100HPLC coupled with a Bruker Essquire quadrupole ion trap-mass spectrometer (QIT-MS, from Bremen, Germany) using electrospray ionization (ESI) in the positive mode for peptide subsample analysis. Additional structural analysis of procyanidin B2 and procyanidin dimers and trimers was
performed in the negative mode. The HPLC was equipped with a binary pump, a degasser, an automated sample manager, a column-heating unit, fluorescence detector and DAD (all by Agilent Technologies, Santa Clara, CA, USA). The separation was achieved on a Waters XBridge BEH130 C18 column (3.5 µm, 2.1×100 mm) column, including a precolumn. The injection volume was 10 µL of each subsample at a flow rate of 350 µL/min. The column temperature was kept at 30 °C constantly. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The gradient started from 5% B and was kept constant for 2 min, then raised linearly to 40% B within 20 min, and again raise linearly to 60% B in 8 min. The following sharp linear rise to 80% B occurred within 1 min and kept constant for 5 min to rinse the column before balancing for the next injection. The detection was monitored with DAD (λ = 214 nm, 260 nm and 280 nm) and a fluorescence detector (λ<sub>ex</sub> = 280 nm, λ<sub>em</sub>=325 nm).

The ESI operating conditions were as follows: dry temperature 300 °C, dry gas 9.0 L/min, nebulizer 70.0 psi, capillary voltage 4500 V, end plate offset 400 V, trap drive 80.0, capillary exit 200.0 V, capillary exit offset 135.0 V, skimmer 1 65.0 V, skimmer 2 20.0 V, lens 1 -2.0V, lens 2 -50.0V, octupole 4.5V, octupole Δ 2.7 and octupole rf amplitude 220.0 Vpp. The mass spectra were collected in the m/z range of 200-2000 in full scan mode and processed with DataAnalysis software.

3.3 Results

3.3.1 Procyanidin dimers and trimers collected from preparative-HPLC

Three samples of aronia juice proanthocyanidins were separated with preparative-HPLC and procyanidins eluted in order of the increasing DP. The first fractions collected in preparative-HPLC were monomers, followed by dimers and trimers (Figure 15). The dimer and trimer fractions were combined after checking the purity with UPLC according to Kylli (2011). The combined dimer and trimer fractions from Aronia 1 involved fractions no 10, 13, 14, 15 and 16 (Figure 15a). In Aronia 2 (Figure 15b), fractions no 11, 14, and 17 were combined. In Aronia 3 (Figure 15c), fractions no 10, 11 and 14 were combined.
3.3.2 Identification and quantification of procyanidin dimers and trimers

Combined procyanidin fractions from preparative-HPLC were identified and quantified by NP-HPLC. Both PDA and fluorescence detectors were employed. Procyanidins are characterised by the responses of both UV absorbance at 280 nm and fluorescence. Procyanidins eluted in order of the degree of polymerization in NP-HPLC. In Figure 16, the two high peaks (1 and 2) are procyanidin monomers. The compounds in peak 6 originated from procyanidin dimers and trimers, which can be determined from the spectra from PDA. In PDA spectra, procyanidins are reported to have homogeneous spectra, having a pronounced maximum at 280 nm and with no band broadening beyond 300 nm (Hellström et al. 2007). The spectrum of compound in peak 6 (Figure 17a) appeared to be consistent with that of procyanidin B2 standard (Figure 17b), indicating that the combined prep-HPLC fractions contained units of procyanidin monomers.
Figure 16. NP-HPLC fluorescence chromatogram of combined preparative-HPLC fractions from aonia juice proanthocyanidins.

Figure 17a. NP- HPLC-PDA spectrum of peak 6 in Figure 16.

Figure 17b. NP-HPLC-PDA spectrum of procyanidin B2 standard (Kahila 2014).

Fluorescence was employed to quantify procyanidins for it is more sensitive to procyanidins than PDA (Lazarus et al. 1999). The concentration of dimeric and trimeric procyanidin was 51.88 µg/mL, which was calculated from the peak area of dimeric and trimeric procyanidin and the standard curve of procyanidin B2.

3.3.3 LC-MS identification of procyanidin dimers and trimers

Dimeric and trimeric procyanidins with the mixture concentration of 492 µg/mL were identified with LC-MS in the negative mode after quantification with NP-HPLC. A number of compounds were characterized as procyanidins, which showed the procyanidin-specific UV absorbance at 280 nm and fluorescence response, including compounds 2, 10, 11, 12, 14 and 15 (Figure 18). Among these compounds, compound two generated a small amount of characteristic procyanidin dimer ions m/z 865, with m/z 659 and 681 as
dominant ions (Figure 19A). The ion m/z 681 could be the sodium adduct of the ion m/z 659. Other compounds, however, did not generate characteristic ions of procyanidin dimers (m/z 577) and trimers (m/z 865) (Hellström et al. 2009). Other distinctive peaks in UV and fluorescence, referring to compounds 14 and 15, produced similar prevailing ions, including m/z 527, m/z 569 and m/z 703 (Figure 19). These compounds however, might be fragments of procyanidins or oxidized procyanidins. In extracted ion chromatogram (EIC) of m/z 865, the trimeric procyanidins mainly eluted with compound two. Ions m/z 577 were most probably produced from procyanidin dimers. This compound displayed weak UV absorbance at 280 nm, yet did not have the fluorescence response.

Figure 18. LC-MS chromatogram of dimeric and trimeric procyanidins in the negative mode. A. Base peak chromatogram (BPC) of procyanidin dimers and trimers. B. Fluorescence chromatogram of procyanidin dimers and trimers (Ex. 280 nm & Em. 325 nm). C. UV chromatogram at 280 nm. D. Extracted ion chromatogram of ions m/z 865. E. Extracted ion chromatogram of ions m/z 577.

3.3.4 LC-MS analysis of procyanidin B2 standard

Procyanidin B2 standard (886 µg/mL) was individually analysed in negative and positive and negative mode to obtain procyanidin B2 standard profiles (Structure of procyanidin B2 is shown in Figure 9. In Figures 20 and 21 respectively, procyanidin B2 standard was analysed in negative and positive mode. Significant UV absorbance at 280 nm and fluorescence response appeared at 9.2 min in both modes. Considering the EIC m/z 579 and m/z 577, which were the molecular ions of procyanidin B2 standard in two modes, the retention time of procyanidin B2 was 9.2 min. Whereas the eluates in the positive mode (Figure 21A) were slightly more diverse than the negative mode (Figure 20A), the dominant ions were similar.
Figure 20. LC-MS profile of the procyanidin B2 standard in negative mode. A. Base peak chromatogram of procyanidin B2. B. Fluorescence chromatogram of procyanidin B2 (Ex. 280 nm & Em. 325 nm). C. UV chromatogram at 280 nm. D. EIC of the ion m/z 577. E. Mass spectrum of the compound eluting at 9.2 min in Figure 20A.

In negative mode (Figure 20A), the dominant ion at 9.2 min is m/z 577, which is originated from the deprotonation of procyanidin B2. Other ions such as m/z 289, m/z 425 and m/z 1156 could all be the result of modification of m/z 577 ions.
Figure 21A. LC-MS profile of procyanidin B2 standard in positive mode. A. Base peak chromatogram of procyanidin B2. B. Fluorescence chromatogram of procyanidin B2 (Ex. 280 nm & Em. 325 nm). C. UV chromatogram at 280 nm. D. EIC of the ion m/z 579. E. Mass spectrum of the compound eluting at 9.2 min in Figure 21A.

In positive mode, the dominant ion in procyanidin B2 peak (Figure 21A) is m/z 579, which is the molecular ion in the protonated form \([M+H]^+\). In addition, the sodium adduct ions m/z 601 of procyanidin B2 in the form of \([M+Na]^+\) were also present in considerable amounts. Moreover, a small amount of ions m/z 409, m/z 867, and m/z 1179 were detected, which may have originated from m/z 579.

3.3.5 Peptides collected from preparative-HPLC

Peptide WLAHKALC m/z 936 and peptide LDQWLCEK m/z 1034 were fractionated and collected with preparative-HPLC after tryptic digestion. The EIC of the two peptides displays the target fractions that would be collected (Figure 22). Peptide WLAHKALC
with a m/z of 936, mainly eluted in the last fraction at around 67 min (Figure 22A), while peptide LDQWLCEK with an m/z of 1034, was collected at around 53 min (Figure 22B). The two peptides had different retention times and do not overlap with each other. The separation of the two peptides of interest from other peptides was very well.

**Figure 22.** Preparative-HPLC extracted ion chromatogram of peptide WLAHKALC m/z 936 (A) and peptide LDQWLCEK m/z 1034 (B)

### 3.3.6 Oxidation of Peptide WLAHKALC m/z 936

**Peptides WLAHKALC m/z 936 without procyanidins**

On day 0, two distinctive peaks appear in TIC with the retention time of 16.7 min and 18.3 min, respectively (Figure 23A). The dormant peak in the EIC of m/z 936 (Figure 23F) suggests that the retention time of peptide WLAHKALC is 18.3 min, which is supported by the corresponding fluorescence response and UV absorption at this time. The UV absorbance at 260 nm (Figure 23B) and fluorescence response (Figure 23E) are due to existence of tryptophan (Salminen et al. 2008). The UV absorbance at 280 nm (Figure 23C) reflects the presence of aromatic amino acids, such as tryptophan (Weishaar et al. 2003). The UV absorbance at 214 nm (Figure 23D) indicates the existence of peptide bonds (Kuipers and Gruppen 2007). The dominant ion in compound two (18.3 min) is m/z 958 while in compound one (16.7 min), the molecular ion cannot be determined, which is possibly unidentified oxidation products or other unknown compound. Compounds that
eluted around and after 25 min were impurities from the column, which were revealed by the presence of relevant peaks in the blank injections.

**Figure 23.** LC-MS analysis of peptide WLAHKALC m/z 936 without procyanidins on day 0 in positive mode. A. Total ion chromatogram of peptide WLAHKALC m/z 936. B. UV chromatogram at 260 nm. C. UV Chromatogram at 280 nm. D. UV Chromatogram at 214 nm. E. Fluorescence Chromatogram of peptide WLAHKALC m/z 936 (Ex. 280 nm & Em. 325 nm). F. Extracted ion chromatogram of m/z 936. G. Mass spectrum of compound one in Figure 23A. H. Mass spectrum of compound two in Figure 23A.

During oxidation, the amount of peptide WLAHKALC m/z 936 decreased. In Figure 24a, the peptide peak (18.3 min) in TIC could be seen on day 0 and day 1, but was not apparent on day 7 and 14. The 16.7 min peak, however, disappeared already on day 1 and many small peaks appeared nearby. The UV 214 nm chromatogram shows similar trend, revealing the reduction of peptides.
The oxidation of tryptophan, which is revealed by fluorescence response (Figure 24 b) and UV 260 nm absorbance (Figure 24 c), displays a similar trend in both chromatograms. Tryptophan eluting at 18.4 min decreased and was almost not seen on day 7 and 14. The compound eluting at 16.8 min disappeared already on day 1. Some new compounds appeared, for example, compound eluting at 15.6 min appeared on day 7 and increased. These compounds were very likely to be the oxidation products of tryptophan (Figure 24 b and c).
Figure 24 b. Fluorescence chromatograms (Ex. 280 nm & Em. 325 nm) of the oxidation of peptide WLAHKALC m/z 936 without procyanidins in positive mode. A. Fluorescence chromatogram on day 0. B. Fluorescence chromatogram on day 1. C. Fluorescence chromatogram on day 7. D. Fluorescence chromatogram on day 14.

Figure 24 c. UV 260 nm chromatograms of the oxidation of peptide WLAHKALC m/z 936 without procyanidins in positive mode. A. UV 260 nm chromatogram on day 0. B. UV 260 nm chromatogram on day 1. C. UV 260 nm chromatogram on day 7. D. UV 260 nm chromatogram on day 14.
Peptide WLAHKALC m/z 936 with procyanidins

Procyanidin B2 standard was added with 1:10 ratio to peptide WLAHKALC. On day 0, the UV and fluorescence chromatograms are very similar to those without procyanidins, as well as the mass spectra of the two outstanding peaks in TIC (Figures 23 and 25). Theoretically, procyanidins have the characteristic maximum UV absorbance at 280 nm (Rohr et al. 2000) and fluorescence response with an excitation wavelength of 280 nm and emission wavelength of 325 nm (Kylli et al. 2011). However, at 9.2 min, which is the retention time of procyanidin B2, there is no UV or fluorescence response in Figures 25 D and E.

![Figure 25](image-url)  
**Figure 25.** LC-MS analysis of peptide WLAHKALC m/z 936 with procyanidins on day 0 in positive mode. A. Total ion chromatogram. B. UV Chromatogram at 214 nm. C. UV Chromatogram at 260 nm. D. UV Chromatogram at 280 nm. E. Fluorescence Chromatogram (Ex. 280 nm & Em. 325 nm). F. Extracted ion chromatogram of m/z 936. G. Mass spectrum of compound one in Figure11A. H. Mass spectrum of compound two in Figure 25A.
During oxidation, the total amount of peptide WLAHKALC displayed an overall declining trend. In Figure 26, the m/z 936 peak (18.4 min) in TIC is still recognizable until day 7. The 16.7 min peak disappears and more small peaks appear nearby from day 1.

![Figure 26 a](image1)

**Figure 26 a.** TIC of the oxidation of peptide WLAHKALC m/z 936 without procyanidins in positive mode. A. TIC on day 0. B. TIC on day 1. C. TIC on day 7. D. TIC on day 14.

Similar to the TIC (Figure 26a), tryptophan in the peptide also decreased, which is revealed from the 18.4 min peak in fluorescence and UV 260 nm chromatograms in Figures 26 b and c. The compound eluting at 16.8 min also decreased with oxidation. Unlike in samples without procyanidins, a new compound appeared on day 0 at 17.5 min and increased on day 1, but followed by a reduction on day 7 and 14 (Figure 26 b). On day 7, the compound eluting at 15.6 min started to increase (Figure 26 b and c).
Figure 26b. Fluorescence chromatograms (Ex. 280 nm & Em. 325 nm) of the oxidation of peptide WLAHKALC m/z 936 with procyanidins in positive mode. A. Fluorescence chromatogram on day 0. B. Fluorescence chromatogram on day 1. C. Fluorescence chromatogram on day 7. D. Fluorescence chromatogram on day 14.

Figure 26c. UV 260 nm chromatograms of the oxidation of peptide WLAHKALC m/z 936 with procyanidins in positive mode. A. UV 260 nm chromatogram on day 0. B. UV 260 nm chromatogram on day 1. C. UV 260 nm chromatogram on day 7. D. UV 260 nm chromatogram on day 14.
Comparison of unoxidized peptide between peptide and peptide/procyanidin group

The oxidation of peptides was compared by tracing the evolution of unoxidized peptide with m/z 936 ions (Figure 27). The amount of the unoxidized peptides was evaluated as the sum of ions m/z 936 and their sodium adduct ions m/z 958 (discussed in 3.4.3). The peak area of the molecular ions was calculated after smoothing with 3 points.

![Figure 27. Evolution of unoxidized peptide WLAHKALC within time as monitored by LC-MS.](image)

During oxidation, the evolution of the unoxidized peptide WLAHKALC in both groups followed a similar declining trend, but the starting points differed slightly (Figure 27). In the peptide/procyanidin sample, the amount of peptide decreased slightly from day 0 to day 1, then experienced a dramatic decrease until day 7, followed by a gradual decline. In peptides without procyanidins, the amount was almost constant between day 0 and day 1 and then markedly diminished. The subsequent decrease from day 7 to day 14 was not considerable, since the peptide concentration on day 7 was already very low. In general, the amount of unoxidized peptides was higher in the peptides with procyanidins than without.

Comparison of oxidation products between peptide and peptide/procyanidin group

The most common oxidation product of peptide WLAHKALC is [WLAHKALC+O]. The concentration change of [WLAHKALC+O] was compared between peptide and peptide/procyanidin group. The ion m/z 952, which is the molecular ion of oxidized peptide WLAHKALC, could not be detected. However, the sodium adducts of m/z 952 ions, seen as m/z 974 were measureable. Therefore, the concentration change of the
oxidation products was analysed by tracing the amount of the ion m/z 974 in both peptide and peptide/procyanidin samples (Figure 28).

![Figure 28. Evolution of oxidation products of peptides WLAHKALC within time as monitored by LC-MS.](image-url)

The concentration of the oxidation products in the peptide WLAHKALC displayed a declining tendency, decreasing gradually in the beginning and then dramatically towards day 7. It was not detectable on day 7 and 14. The peptide/procyanidin samples, however, showed a slightly different trend, which increased from day 0 to day 1, followed by a rapid decline and a subsequent moderate reduction. The concentration of oxidation products was overall higher in the peptide/procyanidin samples than the peptide samples except on day 0, when the peptide samples had higher concentration.

Interaction products of peptide WLAHKALC and procyanidin B2 standard

The peaks of peptide WLAHKALC with and without procyanidins were compared in TIC, UV and FL chromatograms. The peak at 17.5 min in the fluorescence chromatogram (Figure 26b) might be originated from the interaction product, but the corresponding compound in TIC (Figure 25 A) does not show dominant ions as the possible molecular ions of the interaction product. New interaction product peak was not observed in total ion chromatograms (Figures 24a and 26a).

### 3.3.7 Oxidation of peptide LDQWLCEK m/z 1034

**Peptide LDQWLCEK m/z 1034 without procyanidins**

On day 0, the response of peptide LDQWLCEK in TIC was not strong which implies the low concentration of the peptide. The extracted ion m/z 1034 showed a corresponding peak one, indicating that the retention time of the peptide was 15.4 min (Figure 29). Compounds
that eluted around and after 25 min were impurities from the column, which were revealed by the presence of relevant peaks in the blank injections.

Figure 29. LC-MS analysis of peptide LDQWLCEK m/z 1034 without procyanidins on day 0 in positive mode. A. Total ion chromatogram of peptide LDQWLCEK. B. Fluorescence Chromatogram of peptide LDQWLCEK m/z 1034 (Ex. 280 nm & Em. 325 nm). C. Extracted ion chromatogram of m/z 1034.

The unoxidized peptide m/z 1034 decreased with oxidation and was not detectable since day 7. (Figure 30)

Figure 30. Oxidation of peptide LDQWLCEK m/z 1034 without procyanidins in positive mode. A. EIC m/z 1034 on day 0. B. EIC m/z 1034 on day 1. C. EIC m/z 1034 on day 7. D. EIC m/z 1034 on day 14.
Peptide LDQWLCEK m/z 1034 with procyanidins

Procyanidin dimers and trimers collected from aronia juice were added to the peptide LDQWLCEK m/z 1034 with 1:10 ratio. Figure 31 shows the LC-MS analysis of peptide LDQWLCEK m/z 1034 with procyanidins on day 0 in the positive mode. The retention time of the peptide is 15.4 min, evidenced by the EIC m/z 1034 (Figure 31 C) and fluorescence chromatogram (Figure 31 B). Fluorescence responses (Figure 31 B) that appeared at 2.3 min, 4.2 min, 7.9 min, 8.6 min and 9.9 min were similar to the separate analysis of procyanidin dimers and trimers (Figure 4), which confirmed the detection of procyanidin dimers and trimers. However, these procyanidins did not form characteristic molecular ions of dimeric (m/z 579) and trimeric (m/z 867) procyanidins (Figure 31 D and E), which were also in accordance with separate analysis of procyanidin dimers and trimers. During the oxidation, the amount of the unoxidized peptide m/z 1034 decreased until undetectable on day 7 (Figure 32).

Figure 31. LC-MS analysis of peptide LDQWLCEK m/z 1034 with procyanidins on day 0 in positive mode. A. Total ion chromatogram of peptide LDQWLCEK. B. Fluorescence Chromatogram of peptide LDQWLCEK m/z 1034 (Ex. 280 nm & Em. 325 nm). C. Extracted ion chromatogram of m/z 1034. D. Extracted ion chromatogram of m/z 867. E. Extracted ion chromatogram of m/z 579.
Comparison of unoxidized peptides between peptide and peptide/procyanidin group

The concentration change of unoxidized peptide LDQWLCEK during oxidation in both samples was compared by monitoring the peak area of the ion m/z 1034 in the extracted ion chromatogram.

In Figure 33, both peptide and peptide/procyanidin samples show decreasing amount of unoxidized peptide LDQWLCEK during oxidation. The starting concentration was similar in both samples, with slightly higher concentration in the peptide/procyanidin sample. The concentration difference was more obvious on day 1, when the peptide/procyanidin group
concentration was higher. However, since day 7, peptide LDQWLCEK was not detected in both samples.

**Oxidation products of peptide LDQWLCEK**

The oxidation products [LDQWLCEK+O] (m/z 1050) in peptide and peptide/procyanidin samples were extracted both in EIC, but they are under the detection limit. The chromatograms are displayed in Figures 34 and 35.

**Figure 34.** EIC m/z 1050 of oxidation products of peptide LDQWLCEK without procyanidins in positive mode. A. EIC m/z 1050 on day 0. B. EIC m/z 1050 on day 1. C. EIC m/z 1050 on day 7. D. EIC m/z 1050 on day 14.

**Figure 35.** EIC m/z 1050 of oxidation products of peptide LDQWLCEK with procyanidins in positive mode. A. EIC m/z 1050 on day 0. B. EIC m/z 1050 on day 1. C. EIC m/z 1050 on day 7. D. EIC m/z 1050 on day 14.
Interaction products of peptide LDQWLCEK and procyanidin dimers and trimers

The peaks and ions of peptide LDQWLCEK with and without procyanidins were compared in TIC, but no new interaction product peak was observed, which might be due to the low concentration.

3.4 Discussion

3.4.1 Identification of dimeric and trimeric procyanidins with LC-MS

Aronia procyanidin dimers and trimers appear in different types and therefore have different retention times, which explain the appearance of many peaks that show the characteristic UV absorbance (280 nm) and fluorescence responses (Figure 18). Dimeric aronia procyanidin exists in a different type with procyanidin B2 dimer, so that it does not have the same retention time with procyanidin B2 standard. Whereas these compounds (peak 2, 10, 11, 12, 14, 15) do not produce typical molecular ions of procyanidin dimers (m/z 577) and trimers (m/z 865) (Hellström et al. 2009), it may be due to the automatic oxidation or the in-source fragmentation that induces fragment ions. The shared fragment ion patterns of these compounds, regarding ions m/z 527, m/z 569 and m/z 703, may be a characteristic attribute for procyanidin dimers and trimers (Figure 19). The trimeric procyanidin, compound 2 in Figure 4, generated ions m/z 659 and 681 as dominant ions, in which m/z 681 could be the sodium adduct of the ion m/z 659 (Figure 19A). The possible formation pathways of ions m/z 659, m/z 527, m/z 569 and m/z 703 are displayed in Figure 36.

Figure 36 a. Possible formation of ion m/z 659 from procyanidin trimer.
Although procyanidin dimers (Figure 9) carry many hydroxyl groups and hydrogens, the elimination of one water molecule is not accidental. It is very likely to happen on the number 4 carbon of the last dihydropyran heterocycle ring. This carbon carries two hydrogen atoms, which is more prone to form double bond with the adjacent hydroxyl group to generate water, compared with other carbons at similar positions that have only one available hydrogen atom. Yet the whole ortho-dihydroxyl phenol group can be released from any of the three monomers, due to the symmetric structure. The elimination of hydroxyl groups is not random, since it is closely related with the scavenging activity of procyanidins (Cai et al. 2006). It has been postulated that the hydroxyl group at the 3’
position of the B ring might influence the antioxidant activity most (Chen et al. 2014). Therefore, the cleavage of hydroxyl groups in Figure 36 is speculated based on the antioxidant activity towards peptide in the experiment.

The ion m/z 659 could be formed by the loss of one flavonoid, five hydroxyl groups and one water molecule from the procyanidin trimer (Figure 36a). The ion m/z 527 may be produced from procyanidin dimer by losing one water molecule and two hydroxyl groups, with a mass shift of Δ50 amu (Figure 36b). The ions m/z 569 and m/z 703 can come from procyanidin trimers after elimination of dihydroxyl phenol and hydroxyl groups or water (Figures 36c and d).

### 3.4.2 Validation of procyanidin analysis with procyanidin B2 standard

The feasibility of procyanidin analysis in positive mode was evaluated with procyanidin B2 standard (886 µg/mL) using LC-MS in both negative and positive modes. In the negative mode, the existence of ions m/z 289 and m/z 425 under the condition where no fragmentation was performed, indicates the occurrence of in-source fragmentation. The ions m/z 425 may be the products of RDA (Retro Diels Alder) fission of the top unit (Figure 37) (Hellström et al. 2007). The ion m/z 289 can be the monomeric procyanidin ion when the interflavonoid bond cleaves.

![Figure 37](image.png)

**Figure 37.** Formation of m/z 425 through Retro Diels Alder (RDA) mechanism (Hellström et al. 2007).

In the positive mode, the ions m/z 427 may develop from the RDA fission and the subsequent loss of one water molecule results in ions m/z 409. The ions m/z 867 may be produced when the procyanidin dimers dissociated in the solvent and released monomer, which joined back to procyanidin B2, resulting in procyanidin trimer. Two procyanidin B2 ions can also link, generating tetramer ions. The analysis with positive mode in this experiment shows that the procyanidin B2 standard has similar retention time and ionization pathway with the negative mode.
Actually, the procyanidins are frequently analysed with negative mode in ESI-MS since they are better detected due to the acidity of phenolic protons (Hammerstone et al. 1999, Es-Safi et al. 2006, Hellström et al. 2007, Rzeppa et al. 2012). In this experiment, however, the peptides needed to be detected in the positive mode, which favoured procyanidins to be analysed also in the positive mode. Moreover, the acid in the mobile phase which is used to enhance efficiency in the negative mode, prefers to form acid/phenolate adduct resulting in more complex spectra interpretation (Gonzalez-Manzano et al. 2006). In addition, analysis of procyanidins in the positive mode has been proved available in many experiments (Määttä-Riihinen et al. 2005, Gonzalez-Manzano et al. 2006, Kylli et al. 2011, Braunlich et al. 2013). In this experiment, the analysis of procyanidin B2 in the positive mode suggests that the peptide and procyanidin analysis performed in the positive mode is feasible.

3.4.3 Procyanidin B2 standard in peptide WLAHKALC m/z936

Procyanidin B2 standard (32µg/mL) was added to peptide WLAHKALC with a 1:10 ratio, but it did not show the corresponding fluorescence response at 9.2 min. This may because of the low concentration of added procyanidin B2 standard, which was below detection limit. It is also possible that the procyanidin B2 formed a complex with the peptide, which was so little amount or did not have fluorescence property.

3.4.4 Sodium adducts of peptide WLAHKALC m/z 936

In the LC-MS chromatograms of the peptide WLAHKALC on day 0 (Figures 23 and 25), the m/z 936 ion was found less dominant than the ion m/z 958, which was very likely the sodium adduct of peptide m/z936 ions. That is, the molecular ion of peptide m/z936 may appear in the form of [M+H]^+, or the proton is replaced by sodium, forming [M+Na]^+, thus developing the m/z 958 ions. The existence of sodium adducts as the molecular ions are very common, since sodium often exists in the instrument and cannot be evaporated. As a result, the total amount of peptide WLAHKALC sample should be calculated as the sum of m/z 936 and m/z 958. Similarly, the amount of oxidation products [WLAHKALC+O] should also take into account the sodium adduct.
3.4.5 Oxidation of peptide WLAHKALC m/z 936

Comparison of unoxidized peptide between peptide and peptide/procyanidin group

The notable decrease in the amount of unoxidized peptides WLAHKALC reveals a sign of oxidation in both samples (Figure 27). The difference of starting concentration between the two groups suggests that the oxidation already started on day 0, which means the peptide sample was a mixture of oxidized peptides and intact ones with different proportions in the two groups. The extracted m/z 936 and m/z958 ions represent the unoxidized peptide, with a retention time of 18.3 min. The higher amount of unoxidized peptides on the starting day in the peptide/procyanidin group implies that the procyanidin is able to protect peptide from oxidation. This antioxidant activity of procyanidins has been demonstrated in various food environments, including bulk oil, emulsions, liposomes, as well as toward LDL (Määttä-Riihinen et al. 2005, Vattem et al. 2005, Heinonen 2007). This antioxidant activity is also shown on day 7 and 14, in which the amount of m/z 936 is higher in the peptide/procyanidin samples. The oxidation was more rapid in the peptide group, since the amount of unoxidized peptides almost dropped to zero on day 7, while that in the peptide/procyanidin group was still detectable on day 14. Whereas, the amount of unoxidized peptides in the peptide group experienced a slight increase between day 0 and day 1 and it seems to contradict to the antioxidant activity of procyanidin. However, this might be explained by further oxidation. Since the oxidation already happened on day 1, it is possible that further oxidation reduced the already oxidized peptides, releasing more unoxidized form of the peptides. As a result, the concentration of unoxidized peptides seems to increase. In contrast, in the peptide/procyanidin samples, in which procyanidins function in protecting already oxidized peptides from further oxidation, the unoxidized peptides decrease. Wang et al (2014) also had similar unoxidized peptide concentration increase in the peptide group during the oxidation of peptides LIVTQTMK of β-lactoglobulin. Conversely, Koivumäki et al. (2012) did not observe the concentration increase with the same peptide under identical condition. Another explanation for the concentration increase on day 1 may be that the heat in the oxidation process helps unsolvable peptides to dissolve since there was dissolving problem in PIPES buffer before oxidation.
Comparison of oxidation products between peptide and peptide/procyanidin group

In Figure 28, the lower concentration of the oxidation product [WLAHKALC +O] on day 0 in the peptide/procyanidin group compared with peptide group may be explained by the rapid radical scavenging activities of procyanidins (Zheng and Wang 2003). In the peptide samples where no procyanidin was present to scavenge the reactive radical, the oxidation products were higher, which corresponded to the lower concentration of unoxidized peptide m/z 936 in Figure 27. On the following days, the [WLAHKALC+O] oxidation products decreased, which indicates changes of original oxidation products into other oxidation products.

The peptide WLAHKALC consists of Trp-Leu-Ala-His-Lys-Ala-Leu-Cys, in which tryptophan, histidine, cysteine and lysine are most prone to be oxidized (Berlett and Stadtman 1997, Guedes et al. 2010, Törnvall 2010). The oxidation product [WLAHKALC +O] could possibly originate from the oxidation of histidine, cysteine and tryptophan in the peptide. Histidine can be oxidized to 2-oxo-histidine, with an additional oxygen (Figure 38) (Törnvall 2010). Similarly, the sulphur centre of cysteine can be easily oxidized to sulfenic acid, by extracting one oxygen atom (Figure 39) (Xu and Chance 2007).

![Figure 38. Formation of 2-oxo-histidine.](image)

![Figure 39. Formation of sulfenic acid from cysteine.](image)

The oxidation of tryptophan in the peptide is revealed in the fluorescence and UV 260 nm response in Figures 24 b, c and 26 b, c. The oxidation products of tryptophan are very
complex. Moreover, tryptophan in the peptide may contribute to the fluctuation of the oxidation trend. The formation of Trp-derived oxidation products is shown in Figure 8.

Since the tryptophan locates in the amino end of the peptide chain, which means the carboxyl group is attached to the adjacent peptide bond, the amino group remains intact. The oxidative attack appears on the pyrrole ring first (Simat and Steinhart 1998), resulting in diastereomer A and B of PIC, and Oia (Kell and Steinhart 1990). The following oxidation of the phenyl moiety will produce the 5-OH-Trp (Simat and Steinhart 1998). PIC A/B, Oia A/B, as well as 5-5-OH-Trp could be the possible [WLAHKALC+O] oxidation products, with an increase of Δ16 amu. Further oxidation of the m/z 952 ions may occur when Oia A/B is oxidized to DiOia A/B by addition of an oxygen atom, which may explain the decline of the oxidation product since day 7 in Figure 28 (Itakura et al. 1994). However, the formation of Tra and KynA are not possible in the case of this experiment when tryptophan is bonded in the peptide. It is also possible that the pyrrole ring of tryptophan breaks, forming NFK, Kyn, and 3-OH-Kyn (Simat and Steinhart 1998). These new products lead to the change of m/z of the oxidation products, so the concentration of ions m/z 952 will decrease.

In Figures 24 b,c and 25 b,c, the compound eluting at 16.8 min is very likely the primary oxidation product, which disappeared during further oxidation. The compound eluting at 15.6 min, which appeared later in oxidation, is probably secondary oxidation products. The compound eluting at 17.5 min (Figure 25b) might be the primary oxidation product, but it is also possible the interaction product between peptides and procyanidins. The identification of these oxidation products requires further analysis of the spectrum of each possible oxidation product, since they all have different characteristic spectra (Salminen and Heinonen 2008).

The higher amount of the [WLAHKALC+O] oxidation products in the peptide/procyanidin group (Figure 28) suggests that procyanidins promote the formation of primary oxidation products while inhibiting further formation of secondary oxidation products, which can be revealed in Figures 24b and 26b. In the peptide/procyanidin sample (Figure 26b), a new compound appears on day 0 at 17.5 min and increase on day 1, which might be promoted by procyanidin B2 standard. The prooxidant and antioxidant activity of procyanidin B2 is in accordance with the results of Salminen’s experiment with procyanidin and tryptophan (Salminen and Heinonen 2008). The higher concentration of unoxidized peptide (Figure 27) implies the overall antioxidant activity of procyanidin.
However, this overall conclusion seems contradictory to some experiment results, which exhibited the effect of procyanidin B2 in promoting tryptophan loss during oxidation (Salminen et al. 2008). This may be due to the different oxidant used in the oxidation model. In this experiment H₂O₂/FeCl₃ was employed while in the experiment Salminen et al (2008), the oxidant was hexanal/FeCl₂. The presence of hexanal/FeCl₂ in the solution could promote oxidation and polymerization of the phenolics, which can affect their binding ability. This bonding or interaction between peptide and phenolics is closely associated with the antioxidant or pro-oxidant activity of phenolics (Van Acker et al. 1996). It is also possible that the ratio of procyanidin to peptide (1:200 to 1:20) is small compared with 1:10 of this experiment, which results in a different type of procyanidin-peptide interaction, rendering pro-oxidant activity of procyanidin.

3.4.6 Oxidation of peptide LDQWLCEK m/z 1034

In Figure 33, the slight higher concentration in the peptide/procyanidin group on day 0 may indicate the rapid radical scavenging activity of procyanidin dimers and trimers, which could also be observed from the considerable concentration difference on day 1. The subsequent concentration decline may be a result of oxidative modification but more likely attributed to cleavage of peptide backbone or degradation and form peptide fragments, which is implied by absence of oxidation product [LDQWLCEK+O] m/z1050 in both groups. However, it is also possible that some new oxidation products appear in other unknown forms. Another explanation could be that the concentration of peptide LDQWLCEK is originally low (73 µg/mL), resulting in even lower concentration of oxidation products that cannot be detected.

The amino acid sequence in the peptide LDQWLCEK is Leu-Asp-Gln-Trp-Leu-Cys-Glu-Lys. The most susceptible amino acid towards oxidation in peptide LDQWLCEK is tryptophan (Törnvall 2010), which is the same amino acid with peptide WLAHKALC. However, the inhibition of tryptophan oxidation with the collected procyanidin dimers and trimers seemed not as significant as that of procyanidin B2 standard (Figure 28 and 33). Whereas, many researches regarding proanthocyanidins have proved that chain polymerization of catechin monomers increase the radical scavenging activity (Plumb et al. 1998, Ursini et al. 2001, da Silva Porto et al. 2003, Idowu et al. 2010, Chen et al. 2014). Our result with procyanidin dimers and trimers do not conflict with this opinion since some of the collected procyanidins may have lost the ortho-dihydroxy phenol groups, which are considered contributing most to the radical scavenging activity (Cai et al. 2006). Likewise,
Ursini et al. (2001) speculated that the interflavanoid linkage increased the electron delocalization ability of the phenyl radical, resulting in greater antioxidant activity. Furthermore, the loss of hydroxyl groups from the collected procyanidin dimers and trimers also makes a difference in radical scavenging activity (Idowu et al. 2010, Chen et al. 2014). Cysteine is another vulnerable amino acid in the peptide LDQWLCEK m/z 1034 to oxidation, which may be oxidized similarly with that in the peptide WLAHKALC m/z 936.

### 3.4.7 Interaction between ALA peptides and procyanidins

The ALA peptides and procyanidins can interact and may result in some forms of complexes. In the experiment of Wang et al (2014), β-lactoglobulin peptides interacted with sanguin H-6 in the metal-catalysed oxidation system and an interaction product was found. In the case of this experiment, the compound eluting at 17.5 min in peptide WLAHKALC (Figure 26b) might be an interaction product, yet the major dominant ions this compound formed did not match that of the interaction product. This may due to the low concentration of the peptide (320 µg/mL) and procyanidins (32 µg/mL), which leads to challenges regarding the detection of interaction products. The molecular ion of the interaction product may be masked by other prominent ions that are higher in concentration. Moreover, the ratio of peptide and procyanidin may also play an important role in the formation of interaction product as shown in Wang’s experiment (2012). Other than concentration and ratio, many factors can influence the interactions, such as temperature, pH, type of protein, type and structure of phenolics (Ozdal et al. 2013). In addition, the m/z of the interaction products may be out of the detection range when they form polymers or they are not ionized, which may explain the absence of interaction products as well.
4 CONCLUSIONS

This experiment studied the oxidation of ALA peptides and their oxidation in the presence of procyanidins with LC-MS in metal-catalysed oxidation system. Procyanidins exhibited antioxidant activities towards the chosen tryptic peptides WLAHKALC m/z 936 and LDQWLCEK m/z 1034.

Procyanidin B2 standard (dimer) was added to peptide WLAHKALC m/z 936 and procyanidin dimers and trimers were added to peptide LDQWLCEK m/z 1034, both with 1:10 ratio. Procyanidin dimers and trimers were collected from aronia juice proanthocyanidins with preparative-HPLC, identified and quantified with UHPLC and NP-HPLC. The further analysis of dimeric and trimeric procyanidins with LC-MS showed the characteristic fluorescence response and UV absorbance at 280 nm, though the molecular ions of dimeric and trimeric procyanidins were missing, which may be due to automatic oxidation or the in-source fragmentation of procyanidins. Dimeric aronia procyanidins are not the same as procyanidin B2 dimer, thus they do not have the same retention time with procyanidin B2 standard. The analysis of the samples was performed in the positive mode of LC-MS. The feasibility of procyanidin analysis in the positive mode was validated with procyanidin B2 in both negative and positive modes.

Peptide WLAHKALC, which was represented by ion m/z 936 and its sodium adduct m/z 958 decreased during oxidation. The samples with procyanidins had a higher concentration of peptide WLAHKALC than those without procyanidins on the starting day, which revealed the occurrence of oxidation on day 0 and the antioxidant activity of procyanidins. Tryptophan in this peptide is the amino acid most susceptible towards oxidation, as well as cysteine and histidine. The oxidation product [WLAHKALC+O] kept decreasing in peptides samples, but increased on day 1 and decreased afterwards in peptide-procyanidin samples. The decrease of [WLAHKALC+O] indicates their changes into other oxidation products. The fluctuation of the amount of oxidation products in peptide-procyanidin samples could be explained by the procyanidin B2 in the sample, which may promote the formation of primary oxidation product, yet inhibiting further formation of secondary oxidation products. Procyanidin B2 in the peptide-procyanidin sample did not show the corresponding fluorescence response at 9.2 min, which could attribute to the low concentration (32 µg/mL) that is below the detection limit.
Peptide LDQWLCEK m/z 1034 decreased during oxidation and was undetectable on day 7. This concentration decline may be a result of oxidative modification but more likely attributed to cleavage of peptide backbone or degradation and formation of peptide fragments. The amount of unoxidized peptides was higher in peptide-procyanidin samples and the difference was more obvious on day 1, which was due to the antioxidant activity of procyanidin dimers and trimers in the sample. The oxidation product [LDQWLCEK+O] was absent, which may reveal the formation of other new yet unknown oxidation products. It is also possible that the concentration of peptide LDQWLCEK was originally too low for detection of interaction products.

According to the literature, a possible interaction product was supposed to be present yet could not be detected. This may be owing to the low concentration and ratio of peptides and procyanidins. Another possible explanation is that the interaction product is not ionized or the m/z of the interaction product is beyond the detection range. Other factors such as temperature, pH and ratio of peptide and procyanidin may also influence the formation of interaction product.

In conclusion, dimeric and trimeric procyanidins are effective in inhibiting the oxidation of ALA peptides. The oxidation of ALA peptides needs further investigation, such as the oxidation products of tryptophan. For further studies, it is recommended to increase both the concentration and ratio of peptides and procyanidins. The range of MS detection could also be expanded to determine the interaction products.
REFERENCES


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