PEPTIDES AS INHIBITORS OF LIPOXYGENASE AND TYROSINASE

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Peptides as inhibitors of lipoxygenase and tyrosinase

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Abstract

Oxidation reactions catalyzed by enzymes such as lipoxygenase (LOX) and tyrosinase (TYR) initiate food quality decay. Besides their physiological role in the human body, LOX and TYR are involved in certain types of cancer, neurodegenerative diseases and processes of aging. Most common antioxidants able to retard these oxidations, function by scavenging free radicals or by reducing oxidation products formed by these enzymes. The enzyme activity, which is the cause of the formation of these oxidized compounds, remains unaffected. In addition, most of the conventional oxidative enzyme inhibitors are synthetic, unstable, difficult to obtain and cannot be used in food products, cosmetics or medicines from viewpoints of safety and economics. Hydrolyzed proteins from animal and plant sources have been found to possess antioxidant activity. They have a potential to be used as alternative, natural antioxidants. In the research described in this thesis a novel approach was chosen in order to identify protein-based inhibitors for soybean LOX and mushroom TYR. SPOT synthesis was used to synthesize cellulose-bound peptide libraries containing overlapping peptides derived from proteins originating from different industrial sources such as milk, egg, soy, or wheat. Screening of these libraries with a fluorescent labeled LOX or TYR resulted in a set of peptides that specifically bind to these enzymes. The presence of positively charged residues within the peptide sequence appears to be important for interaction with LOX. Preparative synthesis of some binding peptides and subsequent inhibition assays confirmed a true, noncompetitive inhibition of LOX by the octapeptide RINKKIEK from the milk protein β-casein. A substitutional analysis showed that replacement of the glutamic acid residue in RINKKIEK significantly improves binding and inhibition of LOX. The molecular determinants for TYR-inhibiting peptides are different from LOX-inhibiting peptides. Strong TYR-binding peptides always contain one or more arginine residues, often in combination with phenylalanine. Furthermore, the presence of valine, alanine and/or leucine contributes to TYR inhibition. An example of a good TYR-binding and -inhibiting peptide is LFRVASMA from egg ovalbumin, which comprises a combination of these amino acid residues. In conclusion, several inhibitory peptides were identified with potencies comparable to nonpeptidic inhibitors. However, further experiments are required in order to assess the applicability of these antioxidant peptides.
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Chapter 1

General introduction
Bioactive peptides from industrial proteins

Nowadays it is recognized that numerous peptides have specific functional or physiological properties. Such peptides are therefore designated as bioactive peptides. Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health. Nutritionally, proteins are a source of energy and amino acids, which are essential for growth and maintenance. Functionally, proteins contribute to the physicochemical and sensory properties of various protein-rich foods. Furthermore, many dietary proteins possess specific biological properties, which make these components potential ingredients of functional or health-promoting foods (Gill, 1996; Korhonen, 2003). Many of these properties are attributed to physiologically active peptides encrypted in protein molecules. Production of the specific active peptides from industrial protein sources, such as milk, egg, wheat, or soy, allows application in a much broader range of products, in both food and in non-food applications.

From the distribution of the polar and hydrophobic residues on the linear chains of proteins, different fragments can be identified. These fragments are inactive within the sequence of the parent protein, but become active when they are released upon hydrolysis. Hydrolysis of industrial proteins is therefore likely to yield a wide variety of peptides, with a wide range of possible functional and physiological properties. The peptides can be released during food processing, gastrointestinal passage, or in vitro hydrolysis. The activity of these bioactive peptides is based on their inherent amino acid composition and sequence. The size of bioactive peptides may vary from two to as many as twenty amino acid residues, and many peptides are known to have multifunctional properties (Rutherfurd-Markwick, 2005).

Caseins constitute the main protein fraction of milk and are a rich source of bioactive peptides (Floris, 2003; Meisel, 1997; Silva, 2005). Casein-derived peptides have been produced for thousands of years in various fermented milk products. However, it was only realized recently that some of these peptides might exert physiological effects. These beneficial health effects are very diverse in nature and include antihypertensive (FitzGerald, 2004; Vermeirssen, 2004; Yamamoto, 1997), antimicrobial (Hayes, 2006), antioxidative (Rival, 2001a; Suetsuna, 2000b), antithrombotic (Rutherfurd, 2000), immunomodulatory (Cross, 2000), mineral binding (Meisel, 2003), or opioid effects (Teschemacher, 2003). Therefore, caseins and other bulk proteins are increasingly regarded as an important source of bioactive peptides with different physiological properties and functions.
There is increasing commercial interest in the production of bioactive peptides from various sources. Bioactive peptide preparations have the potential to be used as potent drugs having well defined pharmacological effects as mentioned above and in the formulation of functional foods and cosmetics. With the rise of consumer concerns about possible deleterious effects of chemical preservatives and the increasing preference for natural components, protein derived bioactive substances may have value as novel antioxidants in food preservation. The scope of this thesis is the identification and characterization of such peptides derived from industrial proteins, having antioxidant properties that specifically originate from the inhibition of the oxidative enzymes lipoxygenase (LOX) and polyphenol oxidase (PPO).

**Antioxidant peptides and enzyme inhibition**

The term antioxidant is defined as any substance that when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate. An antioxidant can act at different levels in an oxidation sequence. For example, free radical chain reactions may be inhibited either by adding chemicals that retard the formation of free radicals (preventive antioxidants) or by introducing substances that compete with the existing radicals and remove them from the reaction medium (chain-breaking antioxidants).

![Possible mechanisms of antioxidant peptides.](Image)

Proteins from different industrial sources like milk, egg, soy, and wheat have been shown to have antioxidant properties in different (lipid) oxidation systems (Table 1.1). Use of whole protein entities as antioxidant compounds has as a major disadvantage the large amount needed and unfavorable diffusion due to high molecular size. Besides this, it is generally observed that the antioxidant potency of proteins drastically increases upon hydrolysis.
Therefore, research is focusing on peptides and protein hydrolyzates as a source of antioxidative peptides. The mechanisms of antioxidative action of proteins, peptides and individual amino acids include radical scavenging or product reduction, adduct formation, substrate shielding, metal chelating, and enzyme inhibition (Figure 1.1 and Table 1.1). However, the exact mechanism is usually not established, but rather proposed considering both amino acid composition and the observed effect of the antioxidant peptide in a certain oxidation system. These include oxidative stabilization of fats and oils (substrate shielding), iron and/or copper chelation, oxidative enzyme inhibition, and radical scavenging using ABTS•+ (Re, 1999), DPPH• (Schlesier, 2002) or several generated reactive oxygen species (ROS) to assess the antioxidative potency of proteins, hydrolyzates and isolated peptides. The antioxidative effect observed in these systems is related to the presence of certain amino acid residues. The antioxidative effect of individual amino acids has been proposed a few decades ago (Marcuse, 1960). Radical scavenging activities of peptides can be attributed to Cys >>> Trp >> Tyr > Met > His, with Cys being the most potent amino acid residue (Aliaga, 2000; Dávalos, 2004; Meucci, 1997). Both Cys and Met are prone to oxidation and are, in course of action, oxidized to cysteic acid and methionine sulfoxide, respectively (Drozdż, 1988). Trp and Tyr owe their activity to their hydrogen donating ability. The resulting radicals are stabilized by the aromaticity of the side chains. Furthermore, some peptides may act as antioxidants by their ability to chelate metal ions. The basic residues, Lys, Arg and His are able to ligate iron ions (Schneider-Mergener, 1996). His and Cys are both strong metal-coordinating residues and are able to chelate copper and other metal ions (Sóvágó, 2006). Substrate shielding is related to molecular size and is usually observed for high molecular weight fractions or whole proteins (Table 1.1). In case of lipid shielding, both hydrophobic and electrostatic interactions may play an important role in the interaction between the protein and lipid (Kawashima, 1979; Killian, 2000).

The research described in this thesis focuses on direct enzyme inhibition by peptides, since oxidation processes are mainly oxidation cascades that are initiated in a first phase by enzymes. Inhibition of these oxidative enzymes is a more efficient means of reducing the undesired effects of oxidation processes than, for example, scavenging the reaction products generated by these enzymes. Special attention will focus on the physiological role of LOXs and PPOs, the inhibition of these enzymes by peptides and the accompanied implications for food and health.
Table 1.1 – Overview of reported antioxidant proteins, hydrolyzates and peptides from various industrial protein sources.

<table>
<thead>
<tr>
<th>Source</th>
<th>Protein</th>
<th>Treatment</th>
<th>Peptide</th>
<th>Proposed mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine milk</td>
<td>Caseins</td>
<td></td>
<td></td>
<td>Radical scavenging or lipoygenase inhibition</td>
<td>(Laakso, 1982)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Shielding of lipid droplets, radical scavenging, and/or</td>
<td>(Hu, 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>metal chelation</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Trypsin, clostripain, or subtilisin</td>
<td></td>
<td>Radical scavenging</td>
<td>(Rival, 2001a)</td>
</tr>
<tr>
<td>Fermented milk fractions</td>
<td></td>
<td>- α-Casein</td>
<td>Pepsin</td>
<td>YFYPEL</td>
<td>ABTS$^+$ scavenging</td>
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<tr>
<td></td>
<td></td>
<td>- β-Casein</td>
<td>Trypsin</td>
<td>YKEAMAPK VLPVPQK</td>
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<td></td>
<td></td>
<td>- κ-Casein</td>
<td>Lactobacillus delbrueckii</td>
<td>ARHPHPHLSFM</td>
<td></td>
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<tr>
<td>Whey protein</td>
<td>Lactoferrin</td>
<td></td>
<td></td>
<td>DPPH$^+$ scavenging</td>
<td>(Kudoh, 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Shielding of lipid droplets, radical scavenging, and/or</td>
<td>(Hu, 2003)</td>
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<td></td>
<td>metal chelation</td>
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<td></td>
<td></td>
<td>- β-Lactoglobulin</td>
<td>Chymotrypsin</td>
<td>DIQKVAGTWY SLAMAASDISLL</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ROS scavenging and/or Fe$^{3+}$ chelation</td>
<td>(Elias, 2006)</td>
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<tr>
<td>Bovine</td>
<td>BSA</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Pepsin and lysyl endopeptidase</td>
<td>DTHK</td>
<td></td>
<td>(Hatate, 1997)</td>
</tr>
<tr>
<td>Gelatin</td>
<td></td>
<td>Alcalase, pronase E, and collagenase</td>
<td>GEP$^+$GPP$^+$GAP$^{[b]}$</td>
<td>Not discussed</td>
<td>(Kim, 2001b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GPP$^+$GPP$^+$GPP$^{[a]}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GPP$^+$GPP$^+$GPP$^{[a]}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elastin</td>
<td></td>
<td>Pepsin and HCl treatment</td>
<td></td>
<td>Metal chelation and/or interaction with fatty acids</td>
<td>(Hattori, 1998)</td>
</tr>
</tbody>
</table>
Table 1.1 (continued) – Overview of reported antioxidant proteins, hydrolyzates and peptides from various industrial protein sources.

<table>
<thead>
<tr>
<th>Source</th>
<th>Protein</th>
<th>Treatment</th>
<th>Peptide</th>
<th>Proposed mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish</td>
<td>Collagen</td>
<td>Pepsin</td>
<td>LHQPVPE VSQIQVE</td>
<td>•OH scavenging</td>
<td>(Suetsuna, 2004)</td>
</tr>
<tr>
<td>- Gelatin</td>
<td>Alcalase, pronase E, and collagenase</td>
<td></td>
<td>GEP'GPP'GPP'GPP'GPP'GPP'GPP'GPP'GPP'GPP'GPP'GPP'GPP'GPP'GPP'GPP'GPP'GPP'GPP'G&lt;sup&gt;[b]&lt;/sup&gt;</td>
<td>Not discussed</td>
<td>(Kim, 2001a)</td>
</tr>
<tr>
<td>Egg</td>
<td>Egg white</td>
<td>Amano S (&lt;i&gt;Bacillus subtilis&lt;/i&gt;)</td>
<td>AHK VHH VHHANEN</td>
<td>Radical scavenging</td>
<td>(Taguchi, 1988)</td>
</tr>
<tr>
<td>- Albumin</td>
<td>Alcalase</td>
<td></td>
<td></td>
<td>Fe&lt;sup&gt;2+&lt;/sup&gt; chelation</td>
<td>(Tsuge, 1991)</td>
</tr>
<tr>
<td>- Ovalbumin</td>
<td>Heat treatment (&gt;80°C)</td>
<td></td>
<td></td>
<td>O&lt;sub&gt;2&lt;/sub&gt;- scavenging and/or shielding of lipid droplets</td>
<td>(Yamamoto, 1996)</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>Alcalase</td>
<td></td>
<td>YAEERYPI LALAM YQIQL YRGGLEPINF LMSYMWSTSM LELHLRSSHWFSSR</td>
<td>Radical scavenging or interaction with fatty acids?</td>
<td>(Park, 2001)</td>
</tr>
<tr>
<td>Soy</td>
<td>Protein isolate</td>
<td>Pepsin</td>
<td>VNPHDQHQN LVPNHDFQHQN LLPHH LLPHHADADV VPIAYGQLQSGDAVLRAVPSTTY Y</td>
<td>Prooxidant effect&lt;sup&gt;[c]&lt;/sup&gt;</td>
<td>(Yee, 1980)</td>
</tr>
<tr>
<td>- β-Conglycinin</td>
<td>Protease S</td>
<td></td>
<td>VNPHDHQNLVPNHDFQHQQN LLPHH LLPHHADADV VPIAYGQLQSGDAVLRAVPSTTY Y</td>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt; chelation, shielding of lipid droplets, radical scavenging, and/or metal chelation</td>
<td>(Hu, 2003)</td>
</tr>
<tr>
<td>Oat</td>
<td>Gliadin</td>
<td>Pepsin</td>
<td>AQIPQQ</td>
<td>Interaction with fatty acids</td>
<td>(Lehtinen, 2000)</td>
</tr>
<tr>
<td>Wheat</td>
<td>Gliadin</td>
<td></td>
<td></td>
<td>Interaction with fatty acids</td>
<td>(Iwami, 1987; Taguchi, 1988)</td>
</tr>
<tr>
<td>— Zein</td>
<td>Pepsin</td>
<td></td>
<td></td>
<td>Interaction with fatty acids</td>
<td>(Suetsuna, 2002)</td>
</tr>
<tr>
<td>Maize</td>
<td>Zein</td>
<td>Alcalase or papain</td>
<td></td>
<td>Interaction with fatty acids</td>
<td>(Iwami, 1987; Wang, 1991)</td>
</tr>
</tbody>
</table>

[a] Lost upon dephosphorylation; [b] P' = hydroxyproline; [c] Due to presence of lipoxygenase.
Inhibition of lipoxigenases and polyphenol oxidases

LOXs are present in animals and plants where they catalyze the oxidation of polyunsaturated fatty acids (PUFAs) and play a key role in the production of signaling compounds (Farmer, 2001; Funk, 2001) and in the degradation of cellular and intracellular membranes during development (van Leyen, 1998). PPOs are present in animals, plants and fungi where they catalyze the oxidation of phenols in an initial step toward the production of melanin pigments (Costin, 2007). The oxidation processes initiated by these enzymes become deleterious under certain conditions. A number of diseases in humans, different types of cancer and neurodegenerative diseases (Fürstenberger, 2006; Praticò, 2004; Tief, 1998), have been related to an increased level of LOX or PPO activity. Besides the pathological role of these enzymes, LOX and PPO are initiators of uncontrolled oxidation processes that are one of the main causes of food quality decay. LOXs are responsible for the development of rancidity in fat-containing foods (Baysal, 2007) and PPOs initiate browning (Artés, 1998) that occurs during handling and processing of fruits, vegetables, and mushrooms. Generation of off-flavors, loss of nutritional value and loss of economical value of food products are the result. Due to these adverse effects associated with the activity of these enzymes, inhibition of LOXs and PPOs is an important target in both agricultural and pharmaceutical industries. A considerable research effort has therefore been devoted to the identification of appropriate inhibitors for these enzymes (Kim, 2005a; Rescigno, 2002; Schneider, 2005a; Schneider, 2005b).

Most common inhibitors currently known for LOX and PPO are polyphenolic compounds. Polyphenols in general are very powerful chain-breaking antioxidants and able to retard the adverse oxidation reactions initiated by LOX or PPO, but they usually exert their function by radical scavenging or reduction of the oxidation products rather than inhibiting the enzyme. The cause of the formation of these oxidation products is, therefore, not removed. Besides this, chain-breaking antioxidants are consumed during the process, making them less efficient. Lack of specificity usually makes that most LOX and PPO inhibitors are unsuitable for application purposes. For application of such inhibitors as therapeutic drugs, it is important to have a high specificity to induce the clinical effect and to minimize side effects. Specificity is also important when applied as food additives or preservatives, since a low specificity may result in a potential toxic hazard. Therefore, an inhibition resulting from a specific interaction between enzyme and inhibitor is desired in view of many food and non-
food application fields. Since protein-protein interactions can be very specific (Jones, 1996), proteins and peptides may form a good alternative to common inhibitors of LOX and PPO.

**Identification of antioxidant peptides**

*Protein hydrolyzates*

The usual strategy in the identification of antioxidant peptides starts with hydrolysis of proteins that can be easily obtained from natural sources. Whole protein fractions (Fukuzawa, 2005; Hu, 2003), both enzymatically and nonenzymatically prepared protein hydrolyzates (Elias, 2006; Kong, 2006), as well as isolated peptides (Dávalos, 2004; Suetsuna, 2004) have been tested in several assay systems. Once antioxidant activity of a certain hydrolyzate has been detected, a time consuming task begins to unravel the mixture in an attempt to isolate and identify the peptide(s) responsible. Protein hydrolyzates are poorly defined mixtures of amino acids and peptides. The observed effect of a mixture of protein fragments is usually not the result of a single peptide. Apart from synergistic effects (Dávalos, 2004), it is not unlikely that, depending on the type of assay, peptides showing a prooxidant activity exist. This implies that a potential antioxidant peptide can simply be "overlooked" in a complex mixture such as a hydrolyzate.

*Peptide libraries*

An alternative approach to identify and study bioactive peptides, involves screening of synthetic peptide libraries synthesized on solid supports. Solid-phase peptide synthesis (SPPS) was invented by Bruce Merrifield (Merrifield, 1996; Merrifield, 1965). SPPS comprises a series of synthesis cycles, in each of which a protected amino acid is activated and covalently linked to the solid support (first cycle) or previous amino acid (subsequent cycles). The synthesis starts at the C-terminus and proceeds to the N-terminus, exactly the reverse order of protein translation in cells. After each residue is linked to the growing chain, its protective group is removed to allow attachment of the next amino acid. This technique greatly facilitated the preparation of peptides by simply washing the support-bound intermediate products, which enables the use of excess reactants during each cycle, leading to high yields and purity of the final peptide product. This revolution in peptide chemistry was awarded with a Nobel Prize in Chemistry in 1984 and the solid-phase method is now automated and used worldwide in the research on bioactive peptides (Cammish, 2000; Gausepohl, 1992; Houghten, 1991). Initially the peptides were synthesized on resin supports
at a preparative scale. At the beginning of the 1990s, Ronald Frank (Frank, 1992; Frank, 2002) developed the so-called SPOT synthesis of peptide arrays on continuous cellulose membranes at a nanomolar scale. The peptide libraries thus prepared allow rapid screening of a large number of peptides in one single experiment (Figure 1.2). Cellulose-bound peptide libraries have been applied in several molecular interaction studies involving peptide-DNA, peptide-metal, and peptide-protein interactions (Kramer, 1993; Kramer, 1994; Reineke, 2001b; Wenschuh, 2000). Screening of peptide libraries for peptide-metal or peptide-protein interactions is of interest in the identification and optimization of antioxidant peptides with metal-chelating or enzyme-inhibiting properties.

Figure 1.2 – Semi-automated SPOT synthesis in progress (left) and screening of the peptide libraries (right).

Advantages of SPOT synthesis

SPOT synthesis has been used to conduct a number of protein-peptide interaction studies including antibody-antigen recognition for mapping both linear (Hilpert, 2001) and discontinuous epitopes (Reineke, 1996; Reineke, 1999a), receptor-ligand interactions (Bracci, 2001; Reineke, 1998; Reineke, 1999b), enzyme-substrate interactions to determine substrate specificities of proteases (Reineke, 2001a), kinases (Schutkowski, 2004; Schutkowski, 2005) and phosphatases (Espanel, 2002; Espanel, 2005), and enzyme-inhibitor interactions (Hilpert, 2000; Hilpert, 2005; Höhne, 2005; Huang, 2003). These studies demonstrate that the SPOT technique provides a powerful tool in proteomics in general, including the identification and/or optimization of peptides as specific inhibitors of certain enzymes. One aspect of cellulose-bound peptide libraries that should be kept in mind is that the immobilized state of the peptides may affect the molecular interaction studied. In view of oxidative enzyme inhibition, screening of a peptide library for enzyme-peptide interaction offers some advantages over adding protein hydrolyzates to the enzyme catalyzed oxidation reaction. Most important are the high-throughput format of screening and the purity of the
peptides in the library avoiding any interference resulting from other components present in hydrolyzates. Moreover, peptides showing an interaction with the enzyme studied are simply identified by their spot position on the cellulose membrane and need not to be sequenced. These binding peptides are potential inhibitors of the enzyme through a direct interaction with the enzyme. Nonbinding peptides, which may still affect the enzyme catalyzed oxidation reaction via another mechanism than specific enzyme inhibition (Figure 1.1), are excluded. Another advantage of synthetic peptides is that, in theory, any peptide can be prepared. This allows for example a combinatorial approach (Wong, 2004) to identify binding peptides, or a substitutional analysis, with or without the use of unnatural amino acids, to study the molecular interaction on a fundamental level. Since SPOT synthesis of peptide libraries is relatively new and optimization and automation are still under development, there are only a few molecular interaction studies reported using this technique. The research described in this thesis demonstrates a new application of SPOT synthesis in the identification of antioxidant peptides as inhibitors of the oxidative enzymes LOX and PPO.

**Objective and outline of this thesis**

In the past years, research was performed to study the antioxidant properties of proteins, protein hydrolyzates and peptides. Several proteins and peptides have been reported to retard the adverse effects associated with oxidation, however, only little is known about their mechanism of inhibition. Also, structure-activity relationships have not yet been elucidated and the optimal structure of such inhibitory proteins and peptides remains unidentified. In many food and non-food applications, it is desirable to use antioxidant peptides that specifically inhibit the oxidative enzymes LOX and PPO. With the generally applied strategy of protein hydrolysis to identify new inhibitory peptides, it is difficult to distinguish between specific and non-specific inhibition. It is therefore of importance to study the direct interaction between inhibitory peptide and enzyme.

The main objective of the IOP project “New generation of antioxidant peptides from protein hydrolyzates: Isolation and characterisation of peptides as inhibitors for enzymes causing oxidative deterioration of foods” (project IIE00022) was to identify and isolate high added value natural peptides from protein hydrolyzates that have antioxidant properties and specifically inhibit the oxidative enzymes LOX and PPO. To achieve this objective, research focused on the identification of peptides binding to the enzymes, the elucidation of the
mechanism of inhibition of oxidative enzymes by peptides, and the determination of structural requirements for antioxidant and/or enzyme-inhibitory activity of peptides. SPOT synthesis and subsequent screening of peptide libraries was used as an alternative approach to select enzyme-binding peptides as potential inhibitors of LOX and PPO.

This thesis is divided in two parts. Part I deals with the inhibition of LOX. A comprehensive review on LOX activity and the importance of LOX inhibition is given in Chapter 2. Chapter 3 demonstrates the first results of the identification of LOX inhibitory peptides derived from \( \beta \)-casein by using SPOT synthesis and solution-phase inhibition assays. The importance of the amino acid residues within the LOX-inhibiting peptides was assessed via substitutional analysis and the results are reported in Chapter 4. Part II deals with the inhibition of PPO. Analogous to Chapter 2, a review on PPO activity and inhibition is given in Chapter 5. Peptides derived from different industrial protein sources were analyzed for PPO binding and inhibition and the contribution of amino acid residues to both binding and inhibition is described in Chapter 6. Chapter 7 summarizes the main results as described in chapters 3, 4 and 6, combines all newly obtained insights and discusses the future perspectives of these antioxidant peptides.
Part I
Lipoxygenase inhibition
Chapter 2

Inhibition of lipoxygenases by proteins and peptides
Lipoxygenases: occurrence, mechanism and function

LOXs (EC 1.13.11.12) are non-heme, iron-containing dioxygenases that are ubiquitous in plants and animals (Gardner, 1995; Kühn, 1999; Shibata, 1995). The expression levels of different LOXs in tissues vary widely and can differ among the same organism with age, tissue and environmental factors. LOXs catalyze the regio- and enantioselective dioxygenation of polyunsaturated fatty acids (PUFAs) containing a \(1(Z),4(Z)\)-pentadiene system to form hydroperoxide products (Figure 2.1). The most widely accepted reaction mechanism for the LOX-catalyzed peroxidation is radical-based, where the fatty acid is oxidized by the ferric iron within the active site of the enzyme to form a fatty acid radical and a ferrous iron. This radical intermediate is then attacked by dioxygen to form the fatty acid hydroperoxide.

![Proposed catalytic cycle of soybean LOX-1](image)

**Figure 2.1** – Proposed catalytic cycle of soybean LOX-1 (Moody, 2002; Solomon, 2000; Tomchick, 2001) and oxidation of its natural substrate linoleic acid (LA), wherein \(R' = -(CH_2)_7COOH\) and \(R'' = -(CH_2)_4CH_3\). Irreversible hydrogen abstraction appears to be very selective among plant LOXs and occurs only at the double-allelic methylene group C-11 to give rise to a delocalized radical. Subsequently, dioxygen insertion takes place at C-13 to yield a LA peroxyl radical as a catalytic intermediate. Under optimal conditions, LOX-1 from soybean (*Glycine max*) forms mainly \(13(S)\)-hydroperoxy-9(Z),11(E)-octadecadienoic acid.
Soybean lipoxygenases

Research on LOXs showed limited progress until the early 1970s when individual LOX isozymes were first separated and purified from soybean seeds (Axelrod, 1974). Soybean (*Glycine max*) is a rich source of LOXs (Grayburn, 1991). The three isozymes isolated are globular proteins and differ with respect to their isoelectric points, showing values of 5.68, 6.25, and 6.15 for LOX-1, LOX-2, and LOX-3, respectively (Axelrod, 1981). Soybean LOX-1 contains 838 amino acids and has a molecular mass of about 94.0 kD (Shibata, 1987). For LOX-2 and LOX-3 these numbers are 865 amino acids with a molecular mass of 97.0 kD (Shibata, 1988) and 857 amino acids with a molecular mass of 96.7 kD (Yenofsky, 1988). The major differences among the three LOX isozymes can be seen at the level of their activities. LOX-1 has a pH optimum for activity around pH 9.0, while LOX-2 shows a sharp pH maximum at pH 6.5 and LOX-3 displays a broad optimum centered around pH 7. The product of the reaction of linoleic acid (LA) with soybean LOX-1 is almost exclusively 13-hydroperoxy LA. With LOX-2 and LOX-3, roughly equal amounts of the 9- and 13-hydroperoxy products are obtained.

![Figure 2.2 – Regioselective dioxygenation of linoleic acid (LA) and arachidonic acid (AA) by different LOXs. Soybean LOX-1 inserts molecular oxygen at C-13 of LA or at C-15 of AA.](image)

Regioselectivity and classification of lipoxygenases

LOXs are usually classified with respect to the positional selectivity of the dioxygenation of their most common substrates linoleic acid (LA) in plants, and arachidonic acid (AA) in mammals (Feussner, 2002; Kühn, 1999). Although numerous PUFAs or fatty acid-containing molecules can serve as LOX substrates, LA is by far the most widely utilized substrate experimentally. In theory, the dioxygen molecule can add to either end of the pentadiene system. In the case of LA, this leads to two possible products, the 9- and the 13-hydroperoxy LA (Figure 2.2). Accordingly, dioxygenation of LA either at C-9 or C-13 of the carbon
backbone is referred to as 9-LOX or 13-LOX and, for example, dioxygenation of AA at C-15 is referred to as 15-LOX. In plants, 9- and 13-LOX and in mammals, 5-, 8-, 12- and 15-LOX are known. The most extensively studied LOX enzyme is the LOX-1 isoform from soybean, which is a 13-LOX (Figure 2.1). Soybean LOX-1, at pH 9, forms primarily 13-hydroperoxy LA, with a small amount of 9-hydroperoxy LA (van Os, 1979). Oxidation of LA by soybean LOX-1 at pH values below pH 9, increases the amount of 9-hydroperoxy LA (Gardner, 1989). The radical intermediates formed may dissociate from the active site of the enzyme and undergo nonenzymatic reactions to give mixtures of products.

**Stereoselectivity of lipoxygenases**

Insertion of oxygen into the substrate by LOX is not only regioselective but also stereoselective. In theory, both the R and S enantiomer of the 9- and 13-hydroperoxy products of LA can be formed. Hydrogen removal at C-11 by soybean LOX-1 is the rate-limiting step and precedes the oxidation step. This initial hydrogen removal is chirally controlled and occurs on the opposite side of the fatty acid chain where dioxygen insertion takes place. Two models are used to explain the underlying reaction mechanism of positional selectivity of LOXs (Feussner, 2002). Based on data of mammalian LOXs, a space-related hypothesis was established (Borngräber, 1996; Borngräber, 1999; Gillmor, 1997; Kühn, 1990). Here, the AA substrate enters the active site generally with its methyl end first. Then the depth of the substrate-binding pocket determines the site of hydrogen abstraction and the positional selectivity of molecular oxygen insertion depends on this position. However, in plant LOX reactions only one double allylic methylene group in the natural substrate LA seems to be accessible, rendering the space-related hypothesis unlikely. According to a second hypothesis, the substrate orientation is regarded as the key step in the determination of the position of dioxygen insertion (Gardner, 1989). In the case of 13-LOXs, the substrate again enters the active site with its methyl end first. Whereas in 9-LOXs, the substrate is forced into an inverse orientation entering the active site with its carboxyl end first. There has been much debate whether the fatty acid substrate binds carboxyl end first or methyl end first in the active site of soybean LOX-1. A recent study making use of soybean LOX-1 mutants concluded that LA and AA bind carboxyl end first (Ruddat, 2004). However, several studies demonstrated that LA or AA esterified in phosphatidylcholine or triglycerides are oxygenated by soybean LOX-1 similarly to the free fatty acids (Bild, 1977; Luquet, 1993; van Os, 1981). Given the bulkiness of these substrates, oxygenation can occur only via a tail-first entry of the LA into the LOX-1 active site. Another study using an Ala542Gly mutant of LOX-1, which converts LA to both 13(S)- and 9(R)-hydroperoxy LA in a nearly 1 : 1 ratio, showed the same
result (Figure 2.3) (Coffa, 2005). These experiments indicate that the change in stereoselectivity upon Ala542Gly mutation of soybean LOX-1 is not the result of a change in the methyl end first substrate-binding orientation. Reversal of substrate orientation, although less preferred, does occur in the well-characterized double dioxygenation of AA catalyzed by soybean LOX-1 (Schwarz, 1998).

![Figure 2.3 – Proposed active site model of soybean LOX-1 (Coffa, 2005). LA enters the active site with its methyl end first. The reaction to form 13(S)-hydroperoxy LA by soybean LOX-1 proceeds via the stereoselective 11-pro-S hydrogen abstraction. The location of Ala542 opposite to the iron interferes with oxygenation at C-9. This, together with the presence of an oxygen pocket near C-13, ensures oxygenation at C-13 on the opposite side of hydrogen abstraction. The effect of the Ala542Gly mutation is that it opens up space and allows oxygenation at C-9.](image)

**Structure of lipoxygenases**

LOX proteins consist of a single polypeptide chain with a molecular mass of ~94-104 kD in plants and ~75-80 kD in mammals (Brash, 1999). These enzymes are globular molecules that are not glycosylated and do not have any disulfide bridges. LOX proteins contain highly conserved domains and sequence motifs, which are important for the distinct structure and the binding of the catalytic iron. The crystal structures of soybean LOX-1 (Boyington, 1993; Minor, 1993; Minor, 1996; Tomchick, 2001), soybean LOX-3 (Skrzypczak-Jankun, 1997), and rabbit reticulocyte 15-LOX (Choi, 2007; Gillmor, 1997) have been solved. The tertiary structure, which is similar in plant and mammalian LOX, reveals two domains: the N-terminal $\beta$-barrel and a catalytic C-terminal part (Figure 2.4). Based on structural similarities with an analogous C-terminal $\beta$-barrel domain in the mammalian lipases, it has been proposed that the N-terminal $\beta$-barrel of rabbit 15-LOX contains a membrane-docking site and, therefore, may play a critical role in localizing the LOXs near their substrates (Gillmor, 1997). Furthermore, the N-terminal $\beta$-barrel in human 5-LOX resembles C2 domains of calcium-
Lipoxygenase inhibitory peptides

binding proteins and hence it functions in calcium regulation of enzyme activity (Hammarberg, 2000). The catalytic iron is ligated in an octahedral arrangement by three conserved histidines, another histidine (rabbit reticulocyte 15-LOX) or asparagine (soybean LOX-1), and the C-terminal isoleucine. As described above, there is no consensus on how the substrate gains access to the iron cofactor or any definitive information on substrate binding. All three crystal structures are of the inactive ferrous form of the enzyme and there is no apparent entrance to the active site (Wu, 2006). Although the compact structure of soybean LOX-1 in solution does not display any significant difference compared with that from the crystal structure (Dainese, 2005), it is quite likely that movements occur during transition into the active ferric form that will result in opening of the active site cavity (Coffa, 2005). Recently, a reinterpretation of the crystallographic data of 15-LOX showed two different conformations of the molecule, a closed and an open form (Choi, 2007). A new model suggests a catalytic mechanism involving an induced conformational change of the active site binding pocket.

Figure 2.4 – Superposition of the structures of soybean LOX-1 (PDB: 1F8N, light gray) and rabbit 15-LOX (PDB: 1LOX, dark gray). The picture was generated using PyMOL (DeLano, 2002).

**Biological role of plant lipoxygenases**

Lipid peroxidation appears both in developmentally and environmentally regulated processes of plants. The hydroperoxy PUFAs, synthesized by the action of various highly specialized forms of LOXs, are substrates of different enzyme families (Feussner, 2002; Gardner, 1995;
The metabolism of PUFAs via the LOX-catalyzed step and the subsequent reactions are collectively named the LOX pathway. LOX-catalyzed oxidation is an initial step of several pathways that are activated at distinct stages of growth and development and in plant defence reactions upon interaction with pathogens, insects, or abiotic stress (Farmer, 2001; Saravitz, 1996). Signaling compounds such as jasmonates, antimicrobial and antifungal compounds such as leaf aldehydes or divinyl ethers, and a plant-specific blend of volatiles including leaf alcohols are among the numerous products.

**Biological role mammalian lipoxygenases**

Most of our knowledge on LOXs originates from studies of the soybean LOX-1 and other plant LOXs. During the last couple of years more mammalian LOX isoforms were discovered (Kühn, 2002), but for most of them the biological significance remains unclear. The 15-LOX found in red blood cells was the first mammalian enzyme to be well characterized and it appears to be involved in the degradation of mitochondrial membranes during the maturation and differentiation of reticulocytes (Kühn, 1999; Rapoport, 1979; Schewe, 2002). Besides this, 15-LOXs may play a role in organelle degradation during differentiation of the eye lens cell (van Leyen, 1998). Therefore, it is proposed that 15-LOXs are implicated in cell differentiation and development. Among the currently known mammalian LOX isoforms only 12- and 15-LOXs are capable of directly oxygenating ester lipids even when they are integrated within membranes or linked to lipoproteins (Kühn, 2002). Introduction of a polar hydroperoxy group into the hydrophobic tails of fatty acids will alter the noncovalent interactions within the membrane bilayer, which may result in local membrane destabilization. Cell differentiation and/or maturation are characterized by the breakdown of subcellular organelles and remodeling of cellular and subcellular membranes. Membrane remodeling is also involved in trafficking of intracellular vesicles, phagocytosis, degranulation, antigen presentation, and many other processes, and all of them require temporary and local destabilization of the membrane structures, which may be induced by LOX activity. Apart from membrane destabilization, another biological role that can be ascribed to 12- and 15-LOXs may be the formation of bioactive lipids. The more common role of 5-LOXs (Ford-Hutchinson, 1994), 12-LOXs and 15-LOXs in mammalian cells is in the synthesis of hydroperoxy adducts of arachidonic acid, which serve as precursors for the biosynthesis of several important regulatory molecules such as leukotrienes (Funk, 2001) and lipoxins (Aliberti, 2002). In fact, the major products of 12- and 15-LOX pathways are bioactive in cellular and supracellular assay systems (Kühn, 1996). However, it remains to be investigated whether these effects may be of physiological relevance *in vivo*.
Implications of lipid peroxidation in food and health

Negative effects of lipoxygenase activity on health
As long as lipid peroxidation is tightly regulated and restricted to certain cellular compartments, it is an integral part of normal cellular processes described above. Most of the radical intermediates remain enzyme bound, however under certain conditions a considerable proportion of radical intermediates may escape from the active site (Ivanov, 2005; Noguchi, 2002). Excessive lipid peroxidation and the resulting oxidative damage caused to cellular membranes and/or inappropriate production of regulatory molecules is connected to several diseases (Spiteller, 2003). There is much evidence that support the role of LOXs in carcinogenesis (Fürstenberger, 2006; Kudryavtsev, 2005). Several types of cancer usually associated with 12-LOX activity, but also with 5-LOX and 15-LOX, are breast cancer (Natarajan, 1998; Tong, 2002), prostate cancer (Kelavkar, 2001; Matsuyama, 2004; Pidgeon, 2002; Yoshimura, 2005), pancreatic cancer (Ding, 2001; Ding, 2003), gastric cancer (Wong, 2001), bladder cancer (Yoshimura, 2003), and many more. It is proposed that lipid peroxidation promotes tumor growth, metastasis and angiogenesis (Nie, 2002), therefore much research has focused on the development of LOX inhibitors in treatment of cancer (Rioux, 1998; Steele, 1999; Steele, 2000). It should be noted, however, that not all types of LOX are procarcinogenic (Fürstenberger, 2006; Shureiqi, 2001). Some may have anticarcinogenic properties resulting from, for example, LOX-induced apoptosis (Tang, 2002). Besides cancer, other pathological processes have been associated with LOX activity. 5-LOX is involved in the biosynthesis of leukotrienes, which constitute mediators of anaphylactic and inflammatory disorders such as asthma (Nasser, 2002; Silverman, 1998; Young, 1999), psoriasis (Grimminger, 1995; Ikai, 1999) and arthritis (Schiff, 1997). The possible role of 12-LOX and 15-LOX in atherosclerosis is related to their capability of oxygenating PUFAs esterified within complex lipid/protein assemblies, such as biomembranes or lipoproteins (Cathcart, 2000; Feinmark, 1997; Takahashi, 2005). Furthermore, 12-LOX, 15-LOX and 5-LOX may play a role in neurodegenerative diseases such as Alzheimer (Khanna, 2003; Mhater, 2006; Praticò, 2004). Because of all these deleterious effects associated with LOX activity, LOX inhibition is a potential therapy for these diseases (Abe, 2004; Schneider, 2005a; Schneider, 2005b; Whitman, 2002).

Negative effects of lipoxygenase activity in food
Lipid peroxidation not only has a health impact, it is of major importance for the quality of our foods as well. Lipid peroxidation changes the aroma, flavor, taste, texture, color and
nutritional value of fat-containing foods. While some of the reactions are beneficial in bread
making and aroma production, most are deleterious (Baysal, 2007; Eskin, 1977; Robinson,
1995). These changes frequently arise from products formed directly from oxidation of
polyunsaturated lipids. Of even more concern are the free radical intermediates formed that
can interact with and alter other constituents, such as proteins, amino acids, nucleic acids,
carbohydrates, vitamins, and pigments. Oxidation of unsaturated lipids includes free radical
chain reactions that proceed through the generally accepted stages of initiation, propagation
and termination (Scheme 2.1). The enzyme LOX, responsible for a substantial part of lipid
oxidation in raw food sources, is an initiator of peroxidation, but also a propagator. As soon
as ROOH is present, initiation can be furthered nonenzymatically by metal ions (M^{n+} in
Scheme 2.1) or light. Lipid oxidation is of great concern to the food industry and consumers,
because it leads to the development of undesirable off-flavors and potentially toxic reaction
products. Therefore, much research has been done on application of LOX inhibitors and
general antioxidants in foods.

Initiation: \[ \text{LOX}(\text{Fe}^{3+}\text{OH}) + \text{RH} \rightarrow \text{LOX}(\text{Fe}^{2+}\text{OH}_2) + \text{R}^* \]
Propagation: \[ \text{LOX}(\text{Fe}^{2+}\text{OH}_2) + \text{R}^* + \text{O}_2 \rightarrow \text{LOX}(\text{Fe}^{3+}\text{OH}) + \text{ROOH} \]
\[ \text{R}^* + \text{O}_2 \rightarrow \text{ROO}^* \]
\[ \text{ROO}^* + \text{RH} \rightarrow \text{ROOH} + \text{R}^* \]
\[ \text{ROOH} + \text{M}^{n+} \rightarrow \text{RO}^* + \text{OH}^- + \text{M}^{(n+1)+} \]
\[ \text{M}^{(n+1)+} + \text{ROOH} \rightarrow \text{ROO}^* + \text{M}^{n+} + \text{H}^+ \]
\[ \text{RO}^* + \text{RH} \rightarrow \text{ROH} + \text{R}^* \]
Termination: \[ 2 \text{R}^* \rightarrow \text{RR} \]
\[ \text{R}^* + \text{ROO}^* \rightarrow \text{ROOR} \]
\[ \text{R}^* + \text{RO}^* \rightarrow \text{ROR} \]
\[ 2 \text{RO}^* \rightarrow \text{ROOR} \]
\[ 2 \text{ROO}^* \rightarrow \text{ROOR} + \text{O}_2 \]

Scheme 2.1 – General radical chain reactions as a result of LOX mediated initiation of PUFA oxidation.

**Common lipoxygenase inhibitors**

Over the years, numerous inhibitors of lipid peroxidation have been discovered with varying
potency and selectivity. LOX inhibitors can be grouped into redox- and nonredox active
compounds. Redox active compounds are radical scavengers and iron reducing agents.
Nonredox active inhibitors are competitive inhibitors such as substrate or product analogs
(Clapp, 2002; Zhu, 1996), noncompetitive or allosteric inhibitors (Mogul, 2000; Mogul, 2001;
Lipoxygenase inhibitory peptides

Ruddat, 2003) and metal chelators. Most well known LOX inhibitors are phenolic compounds (Table 2.1). The \( IC_{50} \) values reported, or in some cases reduced from reported data, for LOX inhibitors should be considered carefully as they were determined using different assay systems and reaction conditions. In general, the inhibitory activity of phenolic compounds increases with the number of hydroxyl substituents (Laughton, 1991; Sadik, 2003), which suggests that they affect lipid peroxidation through scavenging of the lipid radical intermediates formed during catalysis (Akdemir, 2001). Although the potent inhibitor curcumin may be a strong radical scavenger, it was also found to bind to the active site of LOX (Huang, 1991; Skrzypczak-Jancun, 2000; Skrzypczak-Jankun, 2003). Similarly, structural analysis has revealed that quercetin entrapped within LOX undergoes degradation, which results in a LOX inhibitor, protocatechuic acid, that binds at the active site (Borbulevych, 2004). LOX inhibition studies using propyl gallate and other gallate esters have shown that the \( o \)-dihydroxy moiety, which is also present in protocatechuic acid, is essential for inhibition of LOX (Peterman, 1983). Catechol thus represents the minimum inhibitory structure. Furthermore, the strength of LOX inhibition by gallate esters is directly related to the alkyl chain length, with the most hydrophobic molecules being the most inhibitory. This is also demonstrated by the hydrophobic vitamin E (Chan, 1993a), which is a stronger inhibitor of LOX than trolox (Table 2.1). These findings may explain why nordihydroguaiaretic acid, a hydrophobic molecule containing two \( o \)-dihydroxy moieties, is such a potent LOX inhibitor. The mechanism of inhibition by such molecules is most likely a combination of radical scavenging and binding to the hydrophobic active site of LOX and/or an interaction with the hydrophobic, fatty acid substrate. Not all common inhibitors of LOX contain phenolic hydroxyl groups. For example \( \beta \)-carotene, an effective inhibitor of LOX (Bar-Natan, 1996), does not contain any hydroxyl group. Yet the extended conjugated system of double bonds enables \( \beta \)-carotene to scavenge the linoleyl radical at the beginning of the LOX reaction, leaving the enzyme in the inactive ferrous form (Serpen, 2006). Another exception is zileuton, which represents the first drug of a new treatment for asthma through inhibition of 5-LOX (Wenzel, 1996).

Table 2.1 – Overview of some common inhibitors of several LOXs and reported \( IC_{50} \) values.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Number –OH</th>
<th>( IC_{50} ) / ( \mu M )</th>
<th>Enzyme</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>2</td>
<td>100</td>
<td>LOX-1</td>
<td>(Maccarrone, 1995)</td>
</tr>
</tbody>
</table>
Table 2.1 (continued) – Overview of some common inhibitors of several LOXs and reported $IC_{50}$ values.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Number –OH</th>
<th>$IC_{50}$ / µM</th>
<th>Enzyme</th>
<th>Reference</th>
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<tr>
<td>Baicalein</td>
<td>3</td>
<td>35</td>
<td>LOX-1</td>
<td>(Sadik, 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.64</td>
<td>12-LOX</td>
<td>(Deschamps, 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.12</td>
<td>12-LOX</td>
<td>(Sekiya, 1982a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>15-LOX</td>
<td>(Sadik, 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.6</td>
<td>15-LOX</td>
<td>(Deschamps, 2006)</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>0</td>
<td>300</td>
<td>LOX-1</td>
<td>(Rao, 2002a)</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>2</td>
<td>165</td>
<td>LOX-1</td>
<td>(Rao, 2002b)</td>
</tr>
<tr>
<td>Butylated hydroxyanisole</td>
<td>1</td>
<td>270</td>
<td>LOX-1</td>
<td>(Rao, 2002a; Rao, 2002b)</td>
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<td></td>
<td></td>
<td>46</td>
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<td>(Voß, 1992)</td>
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<td>35700</td>
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<td>(Decker, 1990)</td>
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<tr>
<td>β-Carotene</td>
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<td>2</td>
<td>LOX-1</td>
<td>(Serpen, 2006)</td>
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<td>p-Coumaric acid</td>
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<td>2.5</td>
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<td></td>
<td>30</td>
<td>5-LOX</td>
<td>(Prasad, 2004)</td>
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<td></td>
<td></td>
<td>66</td>
<td>12-LOX</td>
<td>(Jankun, 2006)</td>
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<td>LOX-1</td>
<td>(Naidu, 1995)</td>
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<td>26</td>
<td>5-LOX</td>
<td>(Prasad, 2004; Raghavenra, 2006)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5-LOX</td>
<td>(Neichi, 1983)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.3</td>
<td>5-LOX</td>
<td>(Schneider, 2005b)</td>
<td></td>
</tr>
<tr>
<td>Esculetin</td>
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<td>12-LOX</td>
<td>(Sekiya, 1982b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>12-LOX</td>
<td>(Neichi, 1983)</td>
</tr>
</tbody>
</table>
### Table 2.1 (continued) – Overview of some common inhibitors of several LOXs and reported $IC_{50}$ values.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Number –OH</th>
<th>$IC_{50}$ / µM</th>
<th>Enzyme</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Gossypol</td>
<td>6</td>
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<td>5-LOX</td>
<td>(Hamasaki, 1985)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.7</td>
<td>12-LOX</td>
<td>(Hamasaki, 1985)</td>
</tr>
<tr>
<td>Kojic acid</td>
<td>1</td>
<td>110</td>
<td>LOX-1</td>
<td>(Komoda, 2003)</td>
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<tr>
<td></td>
<td></td>
<td>6.1</td>
<td>LOX-1</td>
<td>(Sircar, 1983)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>LOX-1</td>
<td>(Whitman, 2002)</td>
</tr>
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<td></td>
<td></td>
<td>0.8</td>
<td>5-LOX</td>
<td>(Laughton, 1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>5-LOX</td>
<td>(Voß, 1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28</td>
<td>5-LOX</td>
<td>(Prasad, 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
<td>5-LOX</td>
<td>(Du, 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.1</td>
<td>12-LOX</td>
<td>(Whitman, 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.11</td>
<td>15-LOX</td>
<td>(Whitman, 2002)</td>
</tr>
<tr>
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<td>4</td>
<td>1.7</td>
<td>12-LOX</td>
<td>(Jankun, 2006)</td>
</tr>
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<td>Propyl gallate</td>
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<td>5-LOX</td>
<td>(Laughton, 1991)</td>
</tr>
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<td></td>
<td></td>
<td>2.6</td>
<td>LOX-1</td>
<td>(Takahama, 1985)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.5</td>
<td>LOX-1</td>
<td>(Sadik, 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.3</td>
<td>5-LOX</td>
<td>(Yoshimoto, 1983)</td>
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<td></td>
<td>4</td>
<td>5-LOX</td>
<td>(Laughton, 1991)</td>
</tr>
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<td></td>
<td></td>
<td>25</td>
<td>5-LOX</td>
<td>(Prasad, 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4</td>
<td>15-LOX</td>
<td>(da Silva, 1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>15-LOX</td>
<td>(Sadik, 2003)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>5</td>
<td>0.35</td>
<td>15-LOX</td>
<td>(Schneider, 2005a)</td>
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<tr>
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<td>13</td>
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<td>(Pinto, 1999)</td>
</tr>
<tr>
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<td>63</td>
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<td>(Laughton, 1991)</td>
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<td>170</td>
<td>5-LOX</td>
<td>(Laughton, 1991)</td>
</tr>
</tbody>
</table>
Although many of the compounds mentioned above exhibit potent and specific inhibition of LOXs, they have frequently been associated with a variety of adverse effects due to direct interference with other biological processes in the human body. The redox active inhibitors are typically very potent but of little pharmaceutical value, because of their tendency to produce toxic side effects. The nonredox active inhibitors are more relevant as therapeutic drugs, but their mode of action can be difficult to determine due to the complex kinetics of LOX. In food products antioxidants, such as butylated hydroxyanisole and propyl gallate, may be added to retard lipid oxidation. However, use of synthetic antioxidants in food products is under strict regulation due to the potential health hazards caused by such compounds. Therefore, search for natural antioxidants, such as proteins and peptides, as alternatives to synthetic ones is of great interest among researchers.

### Proteins and peptides as inhibitors of lipid peroxidation

Many proteins, protein hydrolyzates and peptides from different natural sources have been reported to have antioxidant properties (Amarowicz, 1997; Bishov, 1972; Hattori, 1998), but only a few have been related directly to inhibition of lipid peroxidation and even less directly to LOX inhibition. Several mechanisms or combinations thereof may explain the observed inhibitory effect of proteins and peptides on the LOX-catalyzed oxidation of PUFAs. Here follows an overview of proteins and peptides from different sources that were reported to inhibit lipid peroxidation and their proposed mechanisms. Note that most of these proteins and peptides from industrial sources are also listed in Chapter 1 (Table 1.1).

#### Whole proteins

While screening biological fluids for their activity against LOX it was found that milk and milk products were able to inhibit LOX (Laakso, 1982). Further experiments showed that casein, a mixture of proteins of relatively low molecular weight, was the compound responsible for this inhibition. This effect was associated with either trapping of free radicals formed in the
Lipoxygenase inhibitory peptides

reaction, or interaction with the enzyme. Besides casein, other proteins such as BSA (Yukami, 1972), egg white or ovalbumin (Yamamoto, 1996), wheat gliadin (Iwami, 1987), soy protein (Hu, 2003; Pratt, 1972), maize zein (Wang, 1991), and silk sericin (Kato, 1998) have been shown to have antioxidant activities against the peroxidation of lipids or PUFAs. BSA inhibits membrane lipid peroxidation by decreasing the availability of metal catalysts in addition to trapping ROS and free radicals (Fukuzawa, 2005). Also the antioxidant activity of bovine lactoferrin has been attributed to its sequestration of free iron ions (Shimazaki, 2000). The prominent antioxidant effect of egg white and gliadin has been associated with the physical phenomenon of encapsulation of lipid droplets into the hydrophobic regions of the proteins (Taguchi, 1988), thus avoiding oxygen attack. Hydrolyzates of wheat gluten (peptides LQPGQGQQG and AQIPQQ) protect PUFAs against oxidation and also here it is proposed that the hydrophobic amino acids play a role in the interaction between these peptides and fatty acids (Suetsuna, 2002). This protective, antioxidative effect was also observed for soy protein, however in the presence of copper a prooxidative effect was observed (Yee, 1980). It was proposed that soy protein, and hydrolyzates thereof, are able to chelate copper generating complexes that are better catalysts of lipid peroxidation than copper alone. It is therefore important to use different oxidation systems in order to study the antioxidative capacity of proteins. Recently, the antioxidant effects of maize zein was studied in different lipid oxidation systems (Kong, 2006). The results show that the antioxidant activity can be ascribed to a combination of radical scavenging, substrate shielding by accumulation at the lipid/water interface, and metal chelation. These effects were considerably increased upon hydrolysis. Therefore, most research has been focused on the ability of hydrolyzates and individual peptides to inhibit lipid peroxidation rather than whole proteins.

Casein derived peptides

Hydrolyzates of whole casein and β-casein from bovine origin were found to inhibit soybean LOX-1 (Rival, 2001b). The tryptic β-casein digest was the most potent inhibitor of LOX activity and fractionation resulted in the peptide fragments VKEAMAPK, AVYPQR, KVLPPVQK, and VLPVPQK. It was proposed that caseins and casein-derived peptides interact with free radical intermediates in the LOX reaction and hence cause an inhibitory effect against both enzymatic and nonenzymatic lipid peroxidation (Rival, 2001a). These antioxidative casein-derived peptides behave like chain-braking antioxidants reacting with carbon- and oxygen-centered radicals formed in the reaction. All antioxidative peptides were hydrophobic, the most active ones containing two hydrophobic residues at the N-terminal site. The antioxidative peptides identified contain at least one proline, and the most efficient
peptide contains a tyrosine residue. Other peptides with radical scavenging activity identified from casein hydrolyzates are an \( \alpha \)-casein fragment YFYPEL (Suetsuna, 2000b) and a \( \kappa \)-casein fragment ARHPHPHLSFM (Kudoh, 2001).

**L-Carnosine**

L-Carnosine is a \( \beta \)-alanylhistidine dipeptide (Table 2.1), a natural peptide present in large amounts in animal muscle. L-Carnosine is able to inhibit the oxidation of lipids catalyzed by iron, hemoglobin, LOX-1 and singlet oxygen *in vitro* (Chan, 1993b; Decker, 1990; Decker, 1992; Nagasawa, 2001). The hydroxyl radical scavenging and antioxidant activities of L-carnosine and related dipeptides depend on the presence of the peptide bond and amino acid composition of the dipeptides (Chan, 1994). Furthermore, it was demonstrated that the amino acids \( \beta \)-alanine and histidine do not have the same protective properties as L-carnosine (Boldyrev, 1999; Nagasawa, 2001; Wu, 2003a). It is suggested that amphipatic \( N \)-acetylated derivatives of L-carnosine possessing antioxidant properties, are able to suppress the peroxidation of membrane lipids when incorporated into the membranes (Murase, 1993). Similarly, a significantly improved antioxidant effect was observed for \( N \)-acetylated peptides derived from bovine lactoferrin (Wakabayashi, 1999).

**Serum albumin derived peptides**

Other peptides identified show synergism with antioxidants, such as tocopherols, in inhibiting lipid oxidation. Examples are the peptides DTHK and LQHKDDNPNL isolated from hydrolyzates of BSA and HSA, respectively (Hatate, 1997; Hatate, 1998). From this study it appeared that peptides containing the fragment HK are more effective synergists than peptides in which these two residues are isolated from each other. Both peptides showed strong synergism with \( \alpha \)-tocopherol during the autooxidation of linoleic acid and it is proposed that this may be due to the metal-chelating properties of the peptides preventing the consumption of \( \alpha \)-tocopherol.

**Other peptides isolated from skin, gelatin and collagen hydrolyzates**

Gelatin derived peptides from different animal sources containing the repeating motif GPHyp posses antioxidant activity on the peroxidation of linoleic acid (Kim, 2001a; Kim, 2001b). Another peptide SNPEWSWN isolated from a hydrolyzate of cod teiset protein showed radical scavenging activity and synergism with \( \alpha \)-tocopherol (Kim, 2000). Also FKK and FIKK from prawn muscle (Suetsuna, 2000a), mackerel protein hydrolyzate (Chuang, 2000),
Lipoxygenase inhibitory peptides

LHQPVPE and VSQPIQQE from mackerel collagen (Suetsuna, 2004) possess radical scavenging activity.

Egg peptides
Protein extracted from egg yolk was hydrolyzed and two peptides LMSYMWSTSM and LELHKLRSSHWFRR were isolated (Park, 2001). It was proposed that the antioxidative effect of these peptides in a linoleic acid model system may be due to the hydrogen donating ability of tyrosine or the chelating and lipid radical-trapping ability of the imidazole ring of histidine. The peptides AHK, VHH, and VHHANEN were isolated from protease hydrolyzates of egg-white albumin (Tsuge, 1991). Also here it is assumed that the antioxidative effect of the peptides is based on metal chelating activity.

Soy peptides
Antioxidative peptides effective in protection against lipid peroxidation have been isolated from digests of a soybean protein β-conglycinin and synthetic peptides were designed on the basis of the antioxidative peptide LLPHH (Chen, 1995; Chen, 1996; Chen, 1998). Antioxidative peptides showed synergistic effects with nonpeptidic antioxidants, as observed in soybean protein hydrolyzates. Histidine-containing peptides were shown to act as metal chelator, active-oxygen quencher and hydroxyradical scavenger. Histidine and proline in the peptide sequence played important roles in the antioxidative activity, the tripeptide PHH being the most antioxidative. An interesting study using combinatorial peptide libraries based on this tripeptide showed that N- and C-terminal residues of PHH are not as important as the histidine residue in the middle position (Saito, 2003). The tripeptide YHY exerted the highest antioxidative effect in the autooxidation system of LA and a strong synergistic effect in the presence of butylated hydroxyanisole and δ-tocopherol. In another study four peptides, AY, GYY, ADF, and SDF, isolated from a protease hydrolyzate of soy protein showed antioxidant activity against the peroxidation of linoleic acid (Yokomizo, 2002). It is interesting to note that the antioxidative activity of the amino acid mixture of each corresponding peptide was much less than that of each peptide itself. This implies that characteristic amino acid sequences of a peptide are important for the antioxidant activity.

Summary
In summary, both the extent and mode of inhibition of lipid peroxidation by a certain peptide depends on length, amino acid composition, sequence, and the resulting conformation. Despite the fact that many peptides are described, there is not much information about their
efficiency. It appears that more than half of the peptides related to inhibition of lipid peroxidation contain one or more residues of Pro and that about one third of these peptides (not necessarily in combination with Pro) contain at least one Leu, His, Lys or Ala. Despite the fact that only a few of these peptides are described, these frequencies are higher than the average frequencies of these residues normally observed in proteins. Other residues that seem to play an important role are Trp and Tyr. It is not obvious how Pro, Leu, His, Lys, Ala, Trp or Tyr can be related to the antioxidative properties of these peptides. The mode of inhibition can either be radical scavenging, substrate shielding, metal chelation, enzyme inhibition, or a combination of two or more of these mechanisms. The conformation of a peptide is especially important in case of enzyme inhibition, through a direct interaction between the peptide and LOX, in which the presence of Pro in the peptide sequence may contribute to a more rigid conformation (Prajapati, 2007). So far, no evidence of direct LOX inhibition by peptides exists. It is proposed that the hydrophobic nature of Pro, Leu, Ala and other apolar residues may contribute to the interaction between the peptides and fatty acids to protect against oxidation. Peptides may inhibit lipid peroxidation as a result of physicochemical interactions with the membrane lipid bilayer (Walter, 1997). It is more established, however, that peptides containing Trp and Tyr are usually potent inhibitors of lipid peroxidation due to radical scavenging. Similarly, a strong radical scavenging activity was observed for transmembrane proteins (Moosmann, 2000) and peptide hormones (Moosmann, 2002) containing Trp and Tyr. The antioxidative activities of Trp and Tyr may be explained by the capability of indolic and phenolic groups to serve as hydrogen donors. The radical scavenging ability of individual amino acids has been assessed in the ABTS•+ system and other radical-based assay systems and the most potent amino acids are Cys >>> Trp >>> Tyr > Met > His (Aliaga, 2000; Dávalos, 2004; Meucci, 1997). Besides this, His may also be involved in metal chelation. Finally, not only the presence of certain amino acids, but also the peptide linkage between amino acids appears to be important for the observed inhibition. This may either be explained by the simple loss of polarity of amino acids incorporated within peptides and/or by a more specific interaction between the peptides and LOX.
Lipoxygenase inhibitory peptides
Chapter 3

Identification of lipoxygenase inhibitory peptides from β-casein by using SPOT synthesis

Abstract – Lipoxygenases (LOXs) catalyze the oxidation of polyunsaturated fatty acids and play a key role in pathological processes, such as asthma, atherosclerosis and certain types of cancer, and in deterioration of food products. Therefore, inhibition of LOX finds its importance in drug therapy as well as food preservation. Potent LOX inhibitors known today are usually non-specific radical scavengers and, therefore, cannot be used for application purposes. Since protein hydrolysates and individual peptides have been found able to inhibit the LOX catalyzed oxidation, these natural compounds form a good alternative. In this study a novel approach was chosen in order to identify protein based LOX inhibitors. SPOT synthesis was used to synthesize a peptide library containing overlapping β-casein-derived peptides of different length. Screening of this library with fluorescent labeled LOX resulted in peptides that specifically bind to LOX. Most of the binding peptides are derived from five different regions of β-casein. The length of such a peptide, with 8 residues being the most optimal, appears to be important for binding to LOX. Preparative synthesis of some binding peptides and subsequent inhibition assays confirmed a true LOX inhibition by the octapeptide RINKKIEK.
Lipoxygenase inhibitory peptides

Introduction

Lipoxygenases (LOXs) constitute a large family of non-heme iron containing fatty acid dioxygenases, which are widely distributed throughout nature (Brash, 1999; Kühn, 1999). LOXs catalyze the regio- and enantioselective dioxygenation of polyunsaturated fatty acids (PUFAs) containing a 1(Z),4(Z)-pentadiene system, e.g. linoleic acid (LA) in plants or arachidonic acid in mammals. In humans, LOX catalyzes the initial step in a pathway leading to leukotrienes and lipoxins. Increased or inappropriate production of these signal molecules promotes a number of diseases such as asthma (Nasser, 2002), atherosclerosis (Feinmark, 1997), and cancer (Ding, 2001; Kelavkar, 2001; Natarajan, 1998; Steele, 1999). Lipid oxidation can have adverse consequences, not only for human health, but for the quality of foods as well. LOX, responsible for a substantial part of lipid oxidation in foods from animal or vegetal sources, is an initiator of peroxidation. Free lipid radicals can interact with or alter other constituents such as proteins, carbohydrates, vitamins and pigments leading to undesirable changes in aroma, flavor, color and taste.

Much research has been dedicated to the identification of inhibitors that are able to diminish the adverse effects of uncontrolled lipid oxidation. Most potent compounds known today are radical chain-breaking antioxidants rather than true LOX inhibitors. These antioxidants are non-specific, since they randomly react with radical intermediates produced in vivo, and are being consumed during the process. Enzyme inhibitors, however, are specific at the initiation of the radical cascade, hence suppressing radical generation. Hydrolyzed proteins and individual peptides from different industrial sources have been reported to inhibit the LOX catalyzed lipid oxidation: casein, whey, soy and a protein-rich oat fraction (Hu, 2003; Laakso, 1982; Lehtinen, 2000; Rival, 2001a; Rival, 2001b). Besides this, the anti-inflammatory effect of antiflammin 2 (a nonapeptide HDMNKVLDL) can be ascribed to 5-LOX inhibition (Lloret, 1995). Thus, peptides inhibiting LOX have the potential to be used as therapeutic drugs as well as food grade preservatives.

Protein hydrolysates are complex mixtures of amino acids and peptides of which individual effects most probably will interfere with each other. Moreover, not only the preferred LOX inhibition but also several other mechanisms, such as radical scavenging, accumulation of the peptides at the lipid/water interface (Killian, 2000) and changes of the physico-chemical state of the substrate (Lehtinen, 2000), may account for a decreased oxidation rate. Therefore, we have chosen a novel approach using SPOT synthesis (Frank, 1992; Frank,
2002) for the selection of potential LOX inhibitory peptides. The principle of this approach lies in the fact that for a peptide being a LOX inhibitor, it should be able to bind to LOX. Soybean LOX-1 was used as a model enzyme since it is the best characterized LOX, which can be obtained in large quantities from an easy accessible source.

**Experimental procedures**

*SPOT synthesis of the β-casein-derived peptide library*

Peptides based on the β-casein precursor (Entrez Protein accession P02666) were synthesized on a derivatized cellulose membrane (Amino-PEG membrane, substitution 400 nmol cm\(^{-2}\), Intavis AG, Bergisch Gladbach, Germany) using the standard 9-fluorenylmethoxycarbonyl (Fmoc) strategy (Frank, 1992; Frank, 2002). The activated amino acid derivatives were delivered on defined positions on the sheet using a pipetting robot (Autospot upgrade kit for the Automated Multiple Peptide Synthesizer AMS 422, software AutoSpot A, Abimed Analysen-Technik, Langenfeld, Germany). When the desired peptides were fully assembled the membrane was dried and stored under vacuum at –20°C until use.

*Fluorescent labeling of LOX*

Commercial soybean LOX-1 (EC 1.13.11.12, Fluka Chemie, Zwijndrecht, the Netherlands) contains ~35% protein as determined by measurement of \(A_{280}\) compared to albumin (LMW Gel Filtration Kit, Pharmacia USA). SDS-PAGE (12%, Phastsystem Pharmacia) and gel filtration HPLC (TSKgel G2000 SWXL 7.8 mm ID × 30 cm column) analysis showed that no further purification was necessary. A solution of 15 mg mL\(^{-1}\) LOX-1 was labeled with MB (FluoReporter Protein Labeling Kit F-10230, Molecular Probes, Leiden, the Netherlands). The molar ratio of MB to LOX-1 in the reaction mixture was ~15. After purification of the LOX-MB, the average degree of labeling obtained was ~7.6 moles of MB per mole LOX-1. Due to labeling ~30% of the activity of LOX was lost.

*Incubation of cellulose-bound peptides with LOX-MB*

The β-casein-derived peptide library was immersed in 20 mM sodium phosphate pH 7.0 (30 mL) and pre-incubated for 30 min. Then the LOX-MB preparation (500 μL) was added and the library was incubated for one hour while gently shaken. After washing the membrane
three times with 20 mM sodium phosphate pH 7.0 to remove excess LOX-MB, the membrane was illuminated with 366 nm light to reveal the positions of bound LOX-MB.

**SPPS of β-casein-derived peptides**
Peptides selected (Table 3.1) were synthesized on 2-chlorotritylchloride resin (100-200 mesh, substitution 1.04 mmol g⁻¹, Novabiochem, Schwalbach, Germany) using Fmoc chemistry. The coupling steps were accomplished with a pipetting robot (Automated Multiple Peptide Synthesizer AMS 422, software Multiple Peptide ResPrep Version 3.12, Abimed Analysen-Technik, Langenfeld, Germany). The synthesized peptides were purified using HPLC and their sequences were confirmed by MS analysis.

**Inhibition of LOX-catalyzed oxidation of LA**
In a 1 mL quartz cuvet 16.5 mM peptide (25 µL) and 12.5 mM LA in 0.1 M sodium borate pH 9.0 (25 µL, N₂ flushed, containing 1% (v/v) Tween 20) were mixed with 0.1 M Tris-HCl pH 7.4 (725 µL). The reaction was initiated with 45 µg mL⁻¹ LOX (50 µL) and the increase in absorbance at 234 nm, due to the formation of the lipid hydroperoxide, was measured immediately (Perkin Elmer UV/VIS Spectrometer Lambda2S). The initial rate ν was calculated from the steepest linear part of the curve and compared to the control reaction without peptide or standard. The formula used was: ν (µmol min⁻¹) = 825 (µL) × slope (ΔA min⁻¹) / (ε (M⁻¹ cm⁻¹) × 1 (cm)), wherein ε = 25000 M⁻¹ cm⁻¹ (Mogul, 2001). The total volume of the reaction was always 825 µL, containing initially 379 µM LA, 2.7 µg mL⁻¹ LOX, 0.50 mM peptide (if added), and 0.03% (v/v) Tween 20. β-Casein (~20 µM) and L-carnosine (0.50 mM) served as standards.

**Inhibition of AAPH-initiated oxidation of LA**
The AAPH (Acros Organics, Geel, Belgium) initiated oxidation of LA (Peyrat-Maillard, 2003) was performed analogous to the LOX catalyzed oxidation. Yet 2.0 mM AAPH in water (50 µL) was added to start the reaction and the peptides or standards were added during the reaction. The measurements were carried out at 50°C. The percentage of inhibition was calculated from the slope before (control) and after addition of the peptide or standard. During each measurement the reaction rate of the control remained constant. The concentrations of the reactants in the reaction mixture were the same as for the LOX-1 catalyzed oxidation of LA, with the exception of the peptides containing Tyr and Trp residues. Their concentrations were 0.17 and 0.10 mM, respectively.
Results and discussion

**LOX binding by β-casein derived peptides**

Since several peptides derived from β-casein are known to inhibit the LOX catalyzed oxidation of LA (Laakso, 1982; Rival, 2001a; Rival, 2001b), β-casein was selected as a template for SPOT synthesis. In total 367 overlapping peptides derived from β-casein of different length ranging from 10 down to 6 with a frame shift of 3 amino acids, were synthesized on a cellulose membrane. Subsequent incubation of this library with fluorescent, marina blue labeled LOX-1 (LOX-MB) showed preferential accumulation of LOX-MB at certain spots (Figure 3.1). This shows that there is a physical interaction between LOX-MB and particular peptides under the conditions used.

![Figure 3.1](image1.png)

**Figure 3.1** – Negative image of LOX-MB binding to the β-casein-derived peptide library. A dark spot represents binding of LOX-MB. The library consists of overlapping peptides, ranging from 10 down to 6 residues in length, covering the complete sequence of β-casein. Spots in the same relative position to the frame have identical amino acid sequences and differ in length only.

Many LOX-MB binding spots bear peptides that are derived from the same region of β-casein and therefore show strong sequence homology. By translating the data from this experiment into a protein map, five significant regions of β-casein that interact with LOX-MB were identified (Figure 3.2). There are no apparent sequence similarities between any of these
Lipoxygenase inhibitory peptides

regions. Peptides derived from regions 2 and 5 were previously reported to have an inhibitory effect on the LOX catalyzed oxidation of LA (Rival, 2001a; Rival, 2001b). Peptides within the other three binding regions 1, 3 and 4 are new. Interestingly, peptides consisting of 8 amino acid residues interact most effectively with LOX-MB (Figure 3.1 and 3.2). So far, there is no obvious relation between either the size of the different LOX cavities (Minor, 1996) or the length of LA (~20 Å) and the length of the octameric peptides (~25 Å).

![Figure 3.2](image)

**Figure 3.2** – Protein map depicting the regions of the β-casein sequence from where peptides (indicated by bars) show interaction with LOX-MB. The length of a peptide determines the width, position or even the existence of such a region. Binding regions (indicated 1 to 5) – non-binding regions.

**Inhibition of LOX by β-casein derived peptides**

With knowledge about LOX-peptide interactions, the next step was to investigate whether the LOX-binding peptides are able to inhibit LOX. In total 16, both LOX-binding and non-binding, peptides were selected from the β-casein library and synthesized using solid phase peptide synthesis (SPPS) to obtain the free peptides suitable for inhibition assays (Table 3.1). Most of them show a strong interaction with LOX-MB and two of them (43 and 154) served as controls.

In order to discriminate between a true LOX inhibition and a non-specific radical scavenging effect, two LA oxidation systems were used. Besides the LOX catalyzed oxidation of LA, the peptides were also added to a system using 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) to initiate the oxidation of LA. All peptides inhibit to some extent the LA oxidation...
either catalyzed by LOX or AAPH (Figure 3.3). Strikingly, peptides that show a very strong inhibition in the LOX/LA system contain either a Tyr (>40%) or Trp (>60%) residue. These amino acids are not only known to be good radical scavengers, they also have the tendency to accumulate near the lipid carboxyls (Killian, 2000; Meucci, 1997; Moosmann, 2000). In the LOX/LA system, the effect of the radical scavenger L-carnosine as an inhibitor of lipid oxidation (Decker, 1990) is relatively weak compared to Tyr and Trp containing peptides. This can be partially explained by the weaker radical scavenging ability of His, but the large difference in inhibition observed implies that accumulation near the lipid carboxyls of the Tyr and Trp containing peptides might indeed play a role in the LOX/LA assay. Thus, these peptides have a non-specific effect besides the desired LOX inhibition, if present.

Table 3.1 – Sequences of the peptides selected for SPPS and their position on the cellulose membrane. The purity of the peptides was determined by HPLC analysis.

<table>
<thead>
<tr>
<th>Spot number</th>
<th>Region[a]</th>
<th>Sequence</th>
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</tr>
</thead>
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<td>91</td>
</tr>
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<td>QAVPYPQR</td>
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<td>NVPGIEVE</td>
<td>94</td>
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</table>

[a] See Figure 3.2.

In general, the radical scavenging effect of L-carnosine or a peptide is less pronounced in the LOX/LA assay. This may be due to the protection of the formed lipid radicals within the LOX catalytic cleft (Kühn, 2002; Solomon, 2000; Spiteller, 2003). However, under the conditions used in this study, lipid radicals do escape from the active site of LOX. Irganox 1010, a strong radical scavenger, showed an inhibitory on the LOX/LA assay of 23% at a concentration of 0.8 µM. Because of the bulky nature of this compound, the inhibition observed can only be explained by radical scavenging. The use of a radical initiator, such as AAPH in the AAPH/LA system, the lipid radicals generated are free in solution and consequently more subjected to the radical scavenging or hydrogen donating ability of the peptides.
Lipoxygenase inhibitory peptides

By comparison of the results obtained from the inhibition of LOX/LA and AAPH/LA systems by the β-casein-derived peptides, it follows that only two of these peptides, peptide 159 and especially peptide 160, have a stronger inhibitory effect in the LOX/LA system. The radical scavenging activity of peptide 160 is relatively low, indicating that the inhibition observed in the LOX/LA system is a true LOX inhibition. True LOX inhibition is, however, not excluded for the other binding peptides that show inhibitory effects in the LOX/LA system.

![Figure 3.3 – Inhibition (I) of LA oxidation, either catalyzed by LOX or AAPH, by binding and non-binding (43 and 154) peptides and two standards (β-casein and L-carnosine) at pH 7.4 (0.03% (v/v) Tween 20). Inhibition is expressed as a percentage compared to a control reaction with an initial oxidation rate of 14 nmol min⁻¹ for LOX/LA and 2.0 nmol min⁻¹ for AAPH/LA. The concentrations of the peptides and L-carnosine in the reaction were 0.50 mM (unless indicated differently) and the concentration of β-casein was 20 μM.](image)

**LOX kinetics and substrate affinity**

Kinetic analysis of LOX is complicated due to the surfactant nature of the substrate. As in this study, nonionic surfactants such as Tween 20 are usually applied for solubilization of LA in the *in vitro* activity assay. However, it was demonstrated that Tween 20 affects the protein structure of the enzyme and the kinetics of the LOX-catalyzed reaction (Srinivasulu, 1993). At pH 7.4, Tween 20 showed an inhibitory effect on the reaction. With 0.03% Tween 20 an inhibition of about 30% was observed. Therefore, Tween 20 was omitted from the assay and
the kinetics of soybean LOX-1 was analyzed in more detail at pH 7.4 and compared to the kinetics at the reported optimal pH of 9.0 (Began, 1999).

![Figure 3.4](image)

**Figure 3.4** – Oxidation of LA catalyzed by soybean LOX-1 at pH 7.4 (dark line) and pH 9.0 (light line), in \( I = 50 \text{ mM} \) Tris-HCl (without Tween 20), as a function of LA concentration. The Michaelis-Menten curves demonstrate the difference in kinetics below and above the CMC of LA. Insert shows the trace of the hydroperoxide product formed during the reaction with \( v(\text{pH 7.4}) = 21 \text{ nmol min}^{-1} \) and \( v(\text{pH 9.0}) = 13 \text{ nmol min}^{-1} \) at \( c_{\text{LA}} = 98 \mu\text{M} \).

**Table 3.2** – Kinetic parameters of the LOX-catalyzed oxidation below and above the CMC of LA. \( V_{\text{max}} \) values below the CMC are shown between parentheses as these rates are theoretical only.

<table>
<thead>
<tr>
<th>pH</th>
<th>( c_{\text{LA}} / \mu\text{M} )</th>
<th>( K_M / \mu\text{M} )</th>
<th>( V_{\text{max}} / \text{nmol min}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>&lt;74</td>
<td>509 ± 325</td>
<td>(150 ± 88.5)</td>
</tr>
<tr>
<td></td>
<td>&gt;74</td>
<td>38.7 ± 1.80</td>
<td>28.9 ± 0.21</td>
</tr>
<tr>
<td>9.0</td>
<td>&lt;102</td>
<td>11.5 ± 1.50</td>
<td>(14.4 ± 0.76)</td>
</tr>
<tr>
<td></td>
<td>&gt;102</td>
<td>71.6 ± 6.34</td>
<td>22.1 ± 0.42</td>
</tr>
</tbody>
</table>

Without Tween 20 and at substrate concentrations above 40 \( \mu\text{M} \), the rate of the reaction at pH 7.4 was higher than at pH 9.0 (Figure 3.4). Furthermore a lag phase was observed at pH 9.0, which was not present at pH 7.4. More interestingly, the reaction rate suddenly drops a few seconds after initiation of the reaction at pH 7.4. Analysis of the reaction rate as function of the LA concentration demonstrates that this sudden change occurs around 74 \( \mu\text{M} \) LA for pH 7.4 and 102 \( \mu\text{M} \) LA for pH 9.0 (Figure 3.4). This effect can be directly related to the formation of LA micelles, since these LA concentrations correspond to the CMC values.
Lipoxygenase inhibitory peptides

reported for LA (Lagocki, 1976; Mogul, 2000; Mogul, 2001). Note that above the CMC of LA it is no longer the concentration of the monomeric form, but rather the number of LA micelles that is increasing. The kinetic parameters (Table 3.2) show that LOX-1 prefers the micellar form of LA at pH 7.4 and the monomeric form of LA at pH 9.0.

Besides the molecular organization of the substrate, another factor that may influence the enzyme-substrate interaction is the $pK_a$ of LA, albeit that the $pK_a$ itself is strongly dependent on the LA concentration. Due to the presence of multimeric forms of the fatty acid in solution, the $pK_a$ varies from 7.0 to a maximum of 8.0 at high LA concentrations (Glickman, 1995). At pH 7.4 more LA molecules will be protonated, especially at high LA concentrations, than at pH 9.0. Consequently, the majority of the LA micelles will be neutral at pH 7.4, at high LA concentrations, and negatively charged at pH 9.0. Above the CMC of LA, both the substrate affinity as well as $V_{max}$ of LOX-1 is increased at pH 7.4. Assuming that the difference in pH has only a minor effect on the catalytic ability of the enzyme and that the enzyme-substrate interaction largely depends on the charge of the substrate, it appears from the results presented here that LOX-1 prefers neutral micelles over charged micelles. However, an effect of pH on the conformation of the enzyme cannot be excluded. At 40 $\mu$M LA the observed $pK_a$ of LA is just around 7.4 (Glickman, 1995). Thus at LA concentrations significantly below 40 $\mu$M, LA occurs mainly in the negatively charged, monomeric form. In this low substrate concentration range the difference in enzyme-substrate interaction can no longer be explained by the physico-chemical state of the substrate. Therefore, the higher affinity of LOX-1 for LA at pH 9.0 compared to pH 7.4, at low LA concentrations, is most likely the result of conformational changes of LOX-1 induced by an increase in pH (Sudharshan, 2000). The difference in conformation of LOX-1 at higher pH, may explain both the increased affinity for the monomeric form of LA and the lowered (theoretical) $V_{max}$. An increase in LA concentration is accompanied by an increase in the $pK_a$ of LA and it is, therefore, expected that more than 50% of the monomeric form of LA will be protonated just below the CMC of LA at pH 7.4. Since the reaction rate is linear proportional to the concentration of LA between 0 and 74 $\mu$M at pH 7.4, the charge of LA does not seem to affect the reaction rate in this range.

In summary, it is observed that the charge of the monomeric form of LA does not significantly influence the reaction rate. This is supported by a recent finding that the substrate LA enters the LOX-1 active site preferably with its tail first (Coffa, 2005), which implies that the charge of the head group is not relevant for the enzyme-substrate interaction. However at higher...
concentrations of LA, micelles are formed and the negative charge of the substrate starts to play an important role. This can be interpreted as follows. Considering the low pI of LOX-1, LOX-1 has an overall negative charge under the conditions used. The negatively charged head groups of LA at pH 9.0 form a barrier around the surface of the micelle from where LOX-1 is repelled. Consequently, LA molecules incorporated within negatively charged micelles are more protected than within neutral micelles.

**LOX inhibition by binding peptides**

Because of the complex kinetics of LOX-1 demonstrated above, the inhibition kinetics of the peptides on the LOX-1 reaction were performed at lower Tween 20 concentration. Inhibition kinetics of the peptides 159 and 160 showed a mixed-type of inhibition for peptide 159 and a noncompetitive inhibition with $K_i = 146 \pm 24 \, \mu\text{M}$ for peptide 160 (Figure 3.5 and Table 3.3). Peptide 159 shows a mixture between a noncompetitive mechanism of inhibition due to LOX binding, similar to peptide 160, and an apparent competitive mechanism of inhibition due to its radical scavenging ability. The strength of inhibition of these bioactive peptides needs to be improved before they can be applied as natural LOX inhibitors.

![Figure 3.5](image-url)  
*Figure 3.5 – Michaelis-Menten kinetics of the LOX catalyzed formation of LA hydroperoxide at pH 7.4 (0.006% (v/v) Tween 20) in the absence (○) or presence ( ● ) of 50 \mu M peptide 160 (RINKKIEK).*
Lipoxygenase inhibitory peptides

Table 3.3 – Steady-state kinetic parameters of the LOX catalyzed oxidation of LA in presence of peptide 159 (SITRINKK) or peptide 160 (RINKKIEK) obtained from non-linear regression analysis of the experimental data.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$c / \mu M$</th>
<th>$K_M / \mu M$</th>
<th>$V_{max} / \text{nmol min}^{-1}$</th>
<th>$K_i / \mu M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SITRINKK</td>
<td>0</td>
<td>53.9 ± 9.25</td>
<td>27.4 ± 1.27</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>83.9 ± 9.75</td>
<td>25.7 ± 0.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>69.0 ± 15.5</td>
<td>21.9 ± 1.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>105 ± 15.4</td>
<td>21.7 ± 0.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>326 ± 152$^{[a]}$</td>
<td>14.9 ± 3.89</td>
<td></td>
</tr>
<tr>
<td>RINKKIEK</td>
<td>0</td>
<td>66.1 ± 12.9</td>
<td>27.4 ± 1.48</td>
<td>146 ± 23.7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>79.5 ± 20.2</td>
<td>25.6 ± 1.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>68.4 ± 17.7</td>
<td>21.8 ± 1.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>56.8 ± 10.3</td>
<td>19.0 ± 0.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>54.1 ± 6.46</td>
<td>16.3 ± 0.50</td>
<td></td>
</tr>
</tbody>
</table>

[a] Due to solubility problems, the rate $v$ was measured at low LA concentration only.

Conclusions

In this study, peptides derived from five regions of β-casein, of which three are new (Figure 3.2, regions 1, 3 and 4), were found able to bind to LOX. Apart from the Tyr or Trp containing peptides, peptide 160 (RINKKIEK, from region 1) shows the strongest LOX inhibition, with only a minor inhibition on the AAPH induced oxidation of LA. Therefore it can be concluded that the noncompetitive inhibitory effect of RINKKIEK is exclusively due to a true LOX inhibition.

Acknowledgements

This work was supported by the Ministery of Economic Affairs of the Netherlands, the innovation driven program IOP Industrial Proteins (project IIE00022) and by Cargill, Cebeco Egg Research, Cosmoferm BV and DMV International. We thank E.J.M. van Kan for her support in peptide synthesis and J.A. Boeren for performing the MS analyses.
Chapter 4

Improvement of lipoxygenase inhibition by octapeptides

Abstract – The β-casein-derived octapeptide RINKKIEK is a noncompetitive inhibitor of soybean lipoxygenase (LOX). To investigate the molecular determinants for the enzyme-peptide interaction, a peptide library containing substitutional analogs of RINKKIEK was prepared by SPOT synthesis and analyzed for interaction with fluorescent-labeled LOX. The positively charged amino acid residues in RINKKIEK appear to be essential for the LOX-peptide interaction. Replacement of the negatively charged glutamic acid by any other amino acid residue improves LOX binding. For both RINKKIPK and RINKKISK this increase in LOX binding is accompanied by a threefold increase in LOX inhibition.
Lipoxygenase inhibitory peptides

Introduction

Lipoxygenases (LOXs) catalyze the oxidation of polyunsaturated fatty acids (PUFAs) and play a key role in pathological processes and the deterioration of food products. Therefore, inhibition of LOX finds its importance in drug therapy (Matsuyama, 2004; Rioux, 1998; Steele, 1999; Steele, 2000; Yoshimura, 2003; Young, 1999) as well as food preservation (Eskin, 1977; O’Connor, 1991; Robinson, 1995; Whitaker, 1991). Most LOX inhibitors known are radical scavengers containing phenolic hydroxyl groups. Although these compounds are strong LOX inhibitors, they are usually not approved for use in food products. Since protein-protein interactions can be very specific, true inhibition of LOX through direct interaction with proteins or peptides may be more efficient with fewer side effects.

Previous work showed the identification of LOX inhibitory peptides from β-casein (Schurink, 2006). The identification was based on an initial selection of β-casein fragments that are able to bind to LOX followed by LOX inhibition assays with these LOX-binding peptides. Because a true LOX inhibition is most efficient, a distinction was made between LOX-inhibiting peptides with and without radical scavenging activity. The most potent peptides, without radical scavenging activity, were derived from region 1 of β-casein of which RINKKIEK is able to inhibit LOX in a noncompetitive manner (Schurink, 2006). The current paper presents the results obtained from a detailed study toward the improvement of LOX inhibition by these peptides. A peptide library containing substitutional analogs of RINKKIEK was synthesized by using SPOT synthesis (Frank, 1992; Frank, 2002). Both the importance of certain amino acid residues within the peptide as well as potential sequence improvements are described. Soybean LOX-1 was used as a model enzyme, but for application purposes it is interesting to know if the identified inhibitors are able to inhibit a mammalian LOX as well. Therefore the inhibitory effect of the peptides on the activity of rabbit 15-LOX was also determined.

Experimental procedures

SPOT synthesis of the peptide library

Peptides were synthesized on a derivatized cellulose membrane (Amino-PEG membrane, substitution 400 nmol cm⁻², Intavis AG, Bergisch Gladbach, Germany) using the standard 9-fluorenylmethoxycarbonyl (Fmoc) strategy (Frank, 1992; Frank, 2002). The activated amino acid derivatives were delivered on defined positions on the sheet using a pipetting
robot (Autospot upgrade kit for the Automated Multiple Peptide Synthesizer AMS 422, software AutoSpot A, Abimed Analysen-Technik, Langenfeld, Germany). When the desired peptides were fully assembled the membrane was dried and stored under vacuum at −20°C until use.

**Fluorescent labeling of soybean LOX-1**

LOX was labeled with a fluorescent, Marina Blue (MB) dye. Commercial soybean LOX-1 (EC 1.13.11.12, Fluka Chemie, Zwijndrecht, The Netherlands) contains ~72% protein as determined by the Bradford assay (Sigma-Aldrich, Zwijndrecht, The Netherlands). SDS-PAGE analysis showed that no further purification was necessary (Figure 4.1).

![Figure 4.1 – (A) SDS-PAGE of LOX-MB and LOX-1. From left to right: marker proteins (lane 1, molecular weight indicated in kD), LOX-MB (lane 2), and LOX-1 (lane 3). Fluorescence of LOX-MB at 365 nm before staining is shown on the right part of the figure. (B) Absorption spectrum of LOX-MB (dark line) and LOX-1 (light line) at 2.5 mg mL⁻¹. LOX-MB has an absorbance maximum at 365 nm. The molar extinction coefficient of LOX-1 at 280 nm is estimated to be around $44 \times 10^3$ M⁻¹ cm⁻¹.](image)

A solution of LOX-1 in water (10 mg mL⁻¹ protein) was labeled with MB (FluoReporter Protein Labeling Kit F-10230, Molecular Probes, Leiden, The Netherlands). The primary amines within the enzyme are reactive toward the succinimidyl ester moiety of the MB label. The molar ratio of MB to LOX-1 in the reaction mixture was about 15. After separation of the reactants using PD-10 desalting columns (Amersham Biosciences, Uppsala, Sweden) the average degree of labeling of the LOX-MB preparation was about 1 MB label per protein molecule. The MB label did not affect the enzyme activity.
**Incubation of cellulose-bound peptides with LOX-MB**

The peptide library was pre-incubated in 100 mL 50 mM Tris-HCl pH 7.4 (I = 50 mM with NaCl). The background fluorescence of the membrane was recorded using the FluorChem 8800 (Alpha Innotech Corporation, Hoechst Blue HB-500 emission filter 465 nm), before adding 1 mL 2.5 mg mL\(^{-1}\) LOX-MB. The membrane was incubated for about 1 h with LOX-MB at room temperature and after washing 3 times with buffer the fluorescence of the bound LOX-MB was recorded using the same settings as for the background (Figure 4.2). The light density of each spot on the membrane was measured on a linear scale from 0-100%, with 0% = black (no light) and 100% = white (maximum light yield) at an illumination time of 12 s with 365 nm excitation light. The background fluorescence was subtracted to obtain the fluorescence intensity resulting from the bound LOX-MB.

![Negative image of the β-casein-derived peptide library illuminated at 365 nm, (A) before and (B) after incubation with LOX-MB. Binding of LOX-MB to a certain spot results in an increase of intensity.](image)

**LOX tryptophan fluorescence titration measurements**

The LOX-RINKKIEK interaction was studied by monitoring the changes in the Trp fluorescence emission of LOX-1 upon addition of RINKKIEK. In a quartz cuvette 1 mL of 1 μM LOX-1 in 50 mM Tris-HCl pH 7.4 was titrated with a solution of 10 mM RINKKIEK (synthesized previously (Schurink, 2006)) or analogs thereof. Trp fluorescence spectra of LOX-1 were recorded (SPF 500C™ Spectrofluorometer) with 295 nm excitation light 1 min and 10 min after each addition of RINKKIEK solution. The relative fluorescence did not change significantly in time. The fluorescence measured was corrected for dilution. A dissociation constant \( (K_d) \) for the LOX-peptide interaction was derived from the following formula:

\[
\Delta F_{\text{Trp}} = \Delta F_{\text{Trp, max}} / (1 + K_d / c_{\text{peptide}})
\]
Inhibition of LOX-catalyzed oxidation of LA

LOX activity was measured using the following spectrophotometric method. A volume of 25 µL 8.25 mM peptide (Ansynth Service BV, Roosendaal, The Netherlands) and 25 µL 17.9 mM LA (Sigma-Aldrich, Zwijndrecht, The Netherlands) were mixed with 725 µL 50 mM Tris-HCl pH 7.4 (saturated with air) containing 0.03% (v/v) Tween 20 in a 1 mL quartz cuvette thermostated at 25°C. The reaction was initiated with 50 µL 45 µg mL⁻¹ soybean LOX-1 or 300 U mL⁻¹ rabbit 15-LOX (EC 1.13.11.33, Biomol, Plymouth Meeting, USA) and the increase in absorbance at 234 nm, due to the formation of the lipid hydroperoxide, was measured immediately (UV/VIS Spectrometer Lambda2S, Perkin Elmer). The initial rate \( v \) was calculated from the steepest linear part of the curve and compared to a blank reaction without peptide or standard inhibitor. The formula used was: \( v \, (\mu \text{mol min}^{-1}) = 825 \, (\mu \text{L}) \times \text{slope} \, (\Delta A \, \text{min}^{-1}) / (\varepsilon \, (\text{M}^{-1} \text{cm}^{-1}) \times 1 \, (\text{cm})) \), wherein \( \varepsilon = 25000 \, \text{M}^{-1} \text{cm}^{-1} \) (Mogul, 2001). The standard inhibitors used were β-casein (Eurial, Rennes, France), L-carnosine, L-Lys, and a strong radical scavenger propyl gallate. The total volume of the reaction was always 825 µL, containing initially 540 µM LA, 2.7 µg mL⁻¹ LOX-1 or 18 U mL⁻¹ 15-LOX, and 0.25 mM peptide (if added).

Inhibition of AAPH-initiated oxidation of LA

Non-enzymatic oxidation of LA was accomplished by a radical initiator 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) (Peyrat-Maillard, 2003). The AAPH (Acros Organics, Geel, Belgium) initiated oxidation of LA was performed analogous to the LOX-catalyzed oxidation. Yet a solution of 50 µL 20 mM AAPH was added to start the reaction, and the peptides or standards were added during the reaction. The measurements were carried out at 50°C. The concentrations of the reactants in the reaction mixture were the same as for the LOX-catalyzed oxidation of LA.

Results

The LOX-RINKKIEK interaction

A peptide library containing analogs of RINKKIEK was screened for LOX-peptide interaction. In order to detect this interaction, soybean LOX-1 was labeled with a fluorescent MB dye (Figure 4.1). Incubation of the membrane-bound peptide library with LOX-MB and subsequent illumination of the library with UV light revealed the position of the labeled enzyme. The background fluorescence of certain peptides in the library is due to the
Lipoxygenase inhibitory peptides

presence of Trp residues (Figure 4.2A). As does RINKKIEK (Schurink, 2006), most RINKKIEK analogs interact with LOX-MB (Figure 4.2B). The difference in fluorescence intensity before and after incubation of the peptide library with the fluorescent-labeled enzyme is proportional to the amount of LOX-MB binding to a certain peptide.

Peptides derived from region 1 of β-casein having an overlap with RINKKIEK, show a strong interaction with LOX-1 (Figure 4.3). This fine-scan of region 1 showed that especially Lys residues are important for LOX-1 binding. The average fluorescence of LOX-MB binding to all KK containing peptides from region 1 present in the library is 58 ± 11% (n = 21). Note that region 1 is flanked by negatively charged amino acid residues that may have an adverse effect on LOX-1 binding (Figure 4.3).

Figure 4.3 – Fine-scan of β-casein region 1. Relative fluorescence signal (F) of LOX-MB binding to overlapping peptides ranging from nine down to seven residues in length, covering region 1 of β-casein. Sequences of the nonameric peptides (●) are given, sequences of the octameric peptides (□) lack one C-terminal amino acid, and sequences of the heptameric peptides (▲) lack two C-terminal amino acids.

The positive charge of the Lys residues seems to play an important role in the LOX-peptide interaction. The clear relation between the overall charge of a peptide and the LOX-peptide
interaction (Figure 4.4A) indicates the electrostatic nature of the interaction. Maximum LOX-1 binding is observed at an overall charge of +4. The importance of the electrostatic interaction is also demonstrated by the effect of NaCl on LOX-1 inhibition by RINKKIEK (Figure 4.4B). LOX-1 inhibition by this peptide is lowered at higher salt concentration, most probably due to shielding of charged residues, thereby preventing RINKKIEK from binding to LOX-1.

So far the LOX-RINKKIEK interaction has been detected only while RINKKIEK was immobilized on a cellulose membrane. In order to confirm interaction between LOX-1 and the
peptide free in solution, Trp fluorescence titration experiments were carried out. This showed that RINKKIEK, free in solution, interacts with soybean LOX-1 (Figure 4.5). Assuming single-site binding a $K_d$ of $115 \pm 41 \mu M$ can be calculated for the LOX-RINKKIEK complex. This value is in the same range as the $K_i$ of LOX-1 inhibition by RINKKIEK (Schurink, 2006) (Table 4.2). However, the Trp fluorescence quenching profile suggests that multiple binding sites for RINKKIEK exist.

\[
\begin{align*}
F_{\text{Trp}} & = 2.6 \\
F_{\text{Trp}} & = 2.5 \\
F_{\text{Trp}} & = 2.4 \\
F_{\text{Trp}} & = 2.3 \\
F_{\text{Trp}} & = 2.2 \\
\end{align*}
\]

\[
\begin{align*}
c_{\text{RINKKIEK}} / \text{mM} & = 0.1 \\
c_{\text{RINKKIEK}} / \text{mM} & = 0.2 \\
c_{\text{RINKKIEK}} / \text{mM} & = 0.3 \\
c_{\text{RINKKIEK}} / \text{mM} & = 0.4 \\
c_{\text{RINKKIEK}} / \text{mM} & = 0.5 \\
c_{\text{RINKKIEK}} / \text{mM} & = 0.6 \\
c_{\text{RINKKIEK}} / \text{mM} & = 0.7 \\
c_{\text{RINKKIEK}} / \text{mM} & = 0.8 \\
\end{align*}
\]

\[
\begin{align*}
\lambda / \text{nm} & = 310 \\
\lambda / \text{nm} & = 320 \\
\lambda / \text{nm} & = 330 \\
\lambda / \text{nm} & = 340 \\
\lambda / \text{nm} & = 350 \\
\lambda / \text{nm} & = 360 \\
\lambda / \text{nm} & = 370 \\
\lambda / \text{nm} & = 380 \\
\lambda / \text{nm} & = 390 \\
\end{align*}
\]

Figure 4.5 – Binding of RINKKIEK to LOX-1 as monitored by Trp-fluorescence quenching. Relative tryptophan-fluorescence intensity (at 335 nm with 295 nm excitation light) of LOX-1 decreases upon increase of RINKKIEK concentration (left). Tryptophan fluorescence spectrum of LOX-1 in presence of 0 mM (--) and 0.7 mM (---) RINKKIEK (right). The broad emission peak is a result of 14 Trp residues present in LOX-1 (Maccarrone, 2001; Srinivasulu, 2000).

To demonstrate the importance of the RINKKIEK side chains in relation to LOX-1 binding, some unnatural amino acid replacements and an Ala-scan were made (Figure 4.6A). The overlapping peptide SITRINKK (Schurink, 2006), which binds equally well to LOX-1 as RINKKIEK, was analyzed similarly along with some other overlapping peptides that can be obtained by enzymatic hydrolysis of $\beta$-casein region 1 (Figure 4.6B). Again a positive charge of Lys and also Arg is important in the LOX-peptide interaction. Removal of a certain functional group or reducing the side chain to the size of Ala may affect the LOX-binding ability of the peptides significantly. For example, comparison of the results obtained from the unnatural amino acid replacements and the Ala-scan, shows that removal of the $\varepsilon$-amino group from the C-terminal Lys residue, of both RINKKIEK and SITRINKK, has less effect on binding than replacement of the residue by Ala. Apparently, not only the positive charge but also the size of the side chain is important in binding to LOX-1. Removal of all positive charges, within both peptides, significantly reduces LOX-1 binding.
Although Lys appears to be an important residue for LOX-1 binding, polyLys (KKKKKKKKK) is not as good in binding to LOX-1 as RINKKIEK (Figure 4.4A, Figure 4.6 and Table 4.2). This also holds for peptide AAAKKA AK, indicating that the presence of other amino acid residues is important as well. Randomizing the position of the amino acids within RINKKIEK resulted in a set of scrambled peptides. On average, scrambled peptides have a lower affinity for soybean LOX-1, but there is no significant loss of binding (average ∆F of −6.3%). Either
covalently linked to the membrane (Table 4.1) or free in solution (Table 4.2), IRKEIKKN has a slightly lower binding affinity for LOX-1 than RINKKIEK.

Besides RINKKIEK analogs, the library studied contains substitutional analogs of PVPQKAVPYP and QKAVPYPQR. Previous work showed that these and other Tyr-containing peptides have a strong radical scavenging ability and therefore show an inhibitory effect on the LOX-catalyzed oxidation of linoleic acid (LA) (Schurink, 2006). An Ala-scan of both peptides showed that also in these peptides the Lys residues are important for binding to LOX-1. Furthermore, the residues Arg and Tyr seem to be important for LOX-1 binding as well. It is not unlikely that these peptides present two independent mechanisms of inhibition, one being radical scavenging due to the phenolic side-chain of Tyr and the other LOX-1 inhibition through binding. RINKKIEK and its analogs show stronger binding to LOX-1 than the antiflammines 1 (MQMKKVLDLS) and 2 (HDMNKVLDL). These peptides were proposed to be inhibitors of human 5-LOX based on the experimentally measured anti-inflammatory effect that was associated with inhibition of the enzyme (Lloret, 1995). Nevertheless, these peptides show a relatively weak interaction with LOX-1.

Figure 4.7 – Change in LOX-peptide interaction (expressed as $\Delta F$) upon single amino acid replacements within RINKKIEK. Average fluorescence signal due to binding of LOX-MB to RINKKIEK on the membrane is $66.3 \pm 10.6\%$. Replacements made by structurally and/or chemically similar amino acids are grouped and indicated by the same shade.
**Improved LOX binding by RINKKIEK analogs**

To improve the LOX-1 binding affinity of RINKKIEK, a substitutional analysis was carried out. Single-point replacements were made by substituting each residue within the peptide with one of the 19 other natural amino acids (Figure 4.7). The majority of the replacements made to RINKKIEK result in a lower affinity for soybean LOX-1. Nearly any replacement of the N-terminal Arg and the C-terminal Lys results in a significantly weaker LOX-binding affinity. Also, the two central Lys residues of RINKKIEK play an important role in binding to LOX-1. Replacement of a central Lys residue by another amino acid residue (except for Arg) usually weakens LOX-1 binding. Arg and Lys may be interconverted, without affecting LOX-1 binding significantly. Replacements of the neutral amino acids within the RINKKIEK sequence do not improve LOX-1 binding. The weaker binding generally observed (Figure 4.7) implies that the presence of these residues is important as well.

**Table 4.1 – Inhibition (I) of enzymatic and non-enzymatic LA oxidation by RINKKIEK analogs and some standards.** Inhibition is expressed as a percentage compared to a blank reaction with an initial LA oxidation rate of 14 nmol min⁻¹ for soybean LOX-1, 16 nmol min⁻¹ for rabbit 15-LOX, and 4.6 nmol min⁻¹ for AAPH. Relative fluorescence signal (F) of LOX-MB binding to the peptides is given for comparison.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>c / µM</th>
<th>ILOX-1 / %</th>
<th>I15-LOX / %</th>
<th>IAAPH / %</th>
<th>F / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>RINKKIEK</td>
<td>250</td>
<td>42.6 ± 0.92</td>
<td>68.1 ± 1.90</td>
<td>0.00 ± 0.00</td>
<td>66.3 ± 10.6</td>
</tr>
<tr>
<td>RINKKIPK</td>
<td>250</td>
<td>57.7 ± 1.60</td>
<td>57.9 ± 0.49</td>
<td>0.00 ± 0.02</td>
<td>85.9</td>
</tr>
<tr>
<td>RINKKISK</td>
<td>250</td>
<td>59.8 ± 1.30</td>
<td>–69.2 ± 1.46</td>
<td>0.00 ± 0.01</td>
<td>85.0</td>
</tr>
<tr>
<td>IRKEIKKN</td>
<td>250</td>
<td>34.3 ± 0.84</td>
<td>47.2 ± 3.82</td>
<td>0.35 ± 0.00</td>
<td>56.5</td>
</tr>
<tr>
<td>AAKKAALK</td>
<td>250</td>
<td>16.3 ± 0.38</td>
<td>64.2 ± 2.02</td>
<td>0.00 ± 0.01</td>
<td>49.4</td>
</tr>
<tr>
<td>SITRINKK</td>
<td>250</td>
<td>33.9 ± 1.09</td>
<td>65.3 ± 1.99</td>
<td>0.00 ± 0.03</td>
<td>66.9 ± 16.2</td>
</tr>
<tr>
<td>AIBRINKKK</td>
<td>250</td>
<td>42.0 ± 0.92</td>
<td>–31.8 ± 0.32</td>
<td>1.76 ± 0.02</td>
<td>48.9</td>
</tr>
<tr>
<td>KKKKKKKKK</td>
<td>250</td>
<td>73.2 ± 2.11</td>
<td>–30.6 ± 0.51</td>
<td>8.78 ± 0.66</td>
<td>42.1 ± 2.51</td>
</tr>
<tr>
<td>PKYPVEPFTE</td>
<td>250</td>
<td>13.6 ± 0.60</td>
<td>74.6 ± 1.03</td>
<td>19.6 ± 0.38</td>
<td>0.00[a]</td>
</tr>
<tr>
<td>NVPGEIVE</td>
<td>250</td>
<td>4.72 ± 0.10</td>
<td>65.0 ± 0.59</td>
<td>0.00 ± 0.02</td>
<td>0.00[a]</td>
</tr>
<tr>
<td>β-casein</td>
<td>25</td>
<td>37.3 ± 0.98</td>
<td>11.7 ± 0.42</td>
<td>58.2 ± 0.76</td>
<td>-</td>
</tr>
<tr>
<td>L-Lys</td>
<td>250</td>
<td>3.71 ± 0.04</td>
<td>65.9 ± 1.70</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>500</td>
<td>6.07 ± 0.06</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>24.3 ± 0.34</td>
<td>69.8 ± 1.09</td>
<td>3.98 ± 0.04</td>
<td>-</td>
</tr>
<tr>
<td>L-Carnosine</td>
<td>5</td>
<td>0.00 ± 0.01</td>
<td>0.00 ± 0.05</td>
<td>1.85 ± 0.11</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.96 ± 0.05</td>
<td>0.37 ± 0.01</td>
<td>43.4 ± 2.57</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>9.79 ± 0.24</td>
<td>3.56 ± 0.04</td>
<td>65.1 ± 0.98</td>
<td>-</td>
</tr>
<tr>
<td>Propyl gallate</td>
<td>5</td>
<td>32.3 ± 0.82</td>
<td>44.8 ± 3.03</td>
<td>91.7 ± 1.87</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>46.3 ± 1.33</td>
<td>96.0 ± 14.0</td>
<td>95.3 ± 10.8</td>
<td>-</td>
</tr>
</tbody>
</table>

[a] Nonbinding peptides (Schurink, 2006).

Apart from a few replacements of the C-terminal Ile, improvements in LOX-1 binding can be made only by replacement of the negatively charged amino acid residue Glu. A negative charge in the peptide is unfavorable for the LOX-peptide interaction and therefore almost any replacement of Glu results in a stronger LOX-binding ability. In particular replacements by Pro, Met, Ser, Lys, or Phe significantly (ΔF > 15%) improve the LOX-binding affinity of the
Lipoxygenase inhibitory peptides

peptide. For the RINKKIEK analogs RINKKIPK and RINKKISK this improvement in LOX-binding affinity was confirmed in solution (Table 4.2).

Table 4.2 – Steady-state kinetic parameters of the oxidation of LA catalyzed by soybean LOX-1 in presence of inhibitory peptides with the resulting inhibition constants ( \( K_i \) ) and the accompanying dissociation constants ( \( K_d \)) for the LOX-peptide interaction determined from LOX-1 Trp-fluorescence titration experiments.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>( c ) / ( \mu M )</th>
<th>( K_M ) / ( \mu M )</th>
<th>( V_{max} ) / nmol min(^{-1})</th>
<th>( K_i ) / ( \mu M )</th>
<th>( K_d ) / ( \mu M^{[c]} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>RINKKIEK</td>
<td>0.00</td>
<td>157 ± 15.2</td>
<td>27.0 ± 0.77</td>
<td>195 ± 54.8(^{[a]})</td>
<td>115 ± 40.7</td>
</tr>
<tr>
<td></td>
<td>62.5</td>
<td>90.0 ± 20.6</td>
<td>15.2 ± 1.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>84.0 ± 18.6</td>
<td>13.3 ± 1.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>88.8 ± 13.1</td>
<td>11.3 ± 0.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RINKKIPK</td>
<td>0.00</td>
<td>166 ± 17.4</td>
<td>28.6 ± 0.90</td>
<td>71.2 ± 9.24(^{[a]})</td>
<td>73.1 ± 8.76</td>
</tr>
<tr>
<td></td>
<td>62.5</td>
<td>87.1 ± 23.3</td>
<td>12.5 ± 1.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>53.6 ± 8.79</td>
<td>8.63 ± 0.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>32.5 ± 5.30</td>
<td>6.24 ± 0.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RINKKISK</td>
<td>0.00</td>
<td>175 ± 18.8</td>
<td>29.8 ± 0.98</td>
<td>73.6 ± 17.0(^{[a]})</td>
<td>60.8 ± 7.39</td>
</tr>
<tr>
<td></td>
<td>62.5</td>
<td>66.1 ± 7.42</td>
<td>10.6 ± 0.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>52.7 ± 9.44</td>
<td>8.41 ± 0.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>34.0 ± 2.88</td>
<td>6.45 ± 0.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRKEIKKN</td>
<td>0.00</td>
<td>174 ± 20.2</td>
<td>30.1 ± 1.07</td>
<td>192 ± 26.9(^{[a]})</td>
<td>132 ± 17.7</td>
</tr>
<tr>
<td></td>
<td>62.5</td>
<td>109 ± 52.7</td>
<td>19.3 ± 4.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>94.2 ± 38.6</td>
<td>16.5 ± 2.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>75.1 ± 32.7</td>
<td>12.7 ± 2.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SITRINKK</td>
<td>0.00</td>
<td>169 ± 20.7</td>
<td>30.1 ± 1.11</td>
<td>287 ± 107(^{[a]})</td>
<td>137 ± 33.4</td>
</tr>
<tr>
<td></td>
<td>62.5</td>
<td>102 ± 27.2</td>
<td>19.0 ± 2.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>94.0 ± 24.8</td>
<td>16.6 ± 1.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>99.3 ± 18.9</td>
<td>15.4 ± 1.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIBRINKK</td>
<td>0.00</td>
<td>174 ± 20.2</td>
<td>30.1 ± 1.07</td>
<td>162 ± 18.4(^{[a]})</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>62.5</td>
<td>116 ± 33.7</td>
<td>18.5 ± 2.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>107 ± 23.0</td>
<td>16.1 ± 1.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>69.8 ± 21.8</td>
<td>11.5 ± 1.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KKKKKKKK</td>
<td>0.00</td>
<td>204 ± 28.4</td>
<td>31.5 ± 1.41</td>
<td>9.82 ± 1.65(^{[b]})</td>
<td>192 ± 39.3</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>322 ± 37.9</td>
<td>34.6 ± 1.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.33</td>
<td>376 ± 65.3</td>
<td>34.5 ± 2.40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[^{[a]}\] Assuming noncompetitive inhibition; \[^{[b]}\] Competitive inhibition; \[^{[c]}\] Assuming single-site binding.

**Improved LOX inhibition by RINKKIEK analogs**

With knowledge about the importance of individual residues within RINKKIEK in soybean LOX-1 binding, the next step was to determine whether LOX-1 inhibition can be improved accordingly. Therefore the inhibitory effect of the RINKKIEK analogs, on both the LOX-catalyzed oxidation as well as the AAPH-initiated oxidation of LA, was measured along with some other peptides and standards. Two improved LOX-binding peptides, RINKKIPK and RINKKISK, were included and indeed their LOX-inhibiting ability was significantly increased compared to RINKKIEK (Table 4.1). Kinetic experiments showed a LOX-1 inhibition with a \( K_i \)
of 71.2 ± 9.2 µM for RINKKIPK and a $K_i$ of 73.6 ± 17 µM for RINKKISK, compared to a $K_i$ of 195 ± 55 µM for RINKKIEK assuming noncompetitive inhibition (Figure 4.8 and Table 4.2).

The strongest LOX-1 inhibition was observed for KKKKKKKK, a competitive inhibitor of LOX-1 with a $K_i$ of 9.82 ± 1.7 µM. The low radical scavenging effect of KKKKKKKK cannot account for the strong inhibition observed in the LOX/LA assay. An equimolar concentration of free Lys in the assay showed a much weaker inhibition. Clearly the structure of polyLys is required to induce the observed inhibition. Tryptophan titration experiments showed that the interaction between LOX-1 and KKKKKKKK is relatively weak (Table 4.2). This suggests that the strong competitive effect of KKKKKKKK on the LOX-catalyzed oxidation of LA may be due to an interaction with the micelles of the negatively charged substrate. Accumulation of KKKKKKKK at the lipid/water interface may prevent LA from entering the active site and hence cause a competitive inhibitory effect.

![Figure 4.8](image)

**Figure 4.8** – Michaelis-Menten plot of the LOX-catalyzed formation of LA hydroperoxide at pH 7.4 (0.006% (v/v) Tween 20) without inhibitor (○) and in presence of 250 µM RINKKIEK (●) or 250 µM RINKKIPK (●).

For application purposes it is of interest to know whether the designed peptide inhibitors of soybean LOX-1 are able to inhibit mammalian LOX enzymes as well. Therefore the activity of the commercially available rabbit 15-LOX was analyzed in the presence of the RINKKIEK analogs (Table 4.1). RINKKIEK is a good inhibitor of 15-LOX, but there is obviously no correlation between LOX-1 inhibition and 15-LOX inhibition. In some cases, even activation is observed instead of inhibition. The strongest inhibition of 15-LOX was observed for PKYPVEPFTE, a peptide that has no affinity for LOX-1 (Schurink, 2006).
Discussion

Importance of peptide side chains in LOX-1 binding and inhibition

From the previous study with the β-casein-derived peptide library (Schurink, 2006), it has become apparent that the number and order of positive charges in RINKKIEK might play an important role in the LOX-octapeptide interaction. From a fine-scan of β-casein region 1 (Figure 4.3), the relation between overall charge of a peptide and LOX-1 binding (Figure 4.4A), the effect of ionic strength on LOX-1 inhibition (Figure 4.4B), and the (unnatural) amino acid replacements (Figure 4.6) it is evident that the presence of positively charged amino acid residues within the peptide is indeed crucial for the LOX-peptide interaction. Replacement of a neutral amino acid generally results in a lower affinity for LOX-1, indicating that the presence of these residues is important as well (Figure 4.7). Introduction of an additional negative charge within the octapeptide sequence disfavors LOX-1 binding. Similarly, both LOX-1 binding and inhibition is significantly improved upon replacement of Glu within RINKKIEK. Removal of this negative charge results in an overall charge of +4 of the peptide, which appears to be optimal for binding to LOX-1 under the conditions used (Figure 4.4A). These results and the fact that soybean LOX-1 has an overall negative charge at pH 7.4 (Grayburn, 1991), strongly support the electrostatic character of the LOX-peptide interaction.

The order of the residues within the peptide (e.g. +00++0–+ for the charge distribution within RINKKIEK) does not seem to be as relevant as the presence of these residues. This was demonstrated by the scrambled RINKKIEK peptides that all show a similar binding affinity for LOX-1. In addition, the LOX-1 inhibition kinetics of IRKEIKKN (0++–0++0), is similar to the inhibition kinetics of RINKKIEK (Table 4.2). The Trp fluorescence data (Figure 4.5) suggest that there are multiple binding sites with different affinities for RINKKIEK present on LOX-1. Together with the results of the scrambled peptides, this would imply that the LOX-RINKKIEK interaction is not very specific. RINKKIEK can bind to other proteins as indicated by the observed interaction with tyrosinase (Schurink, 2007). Nevertheless, addition of a large excess of negatively charged BSA (23 times more BSA than LOX-1) to the LOX-1 reaction, did not affect the extent of LOX-1 inhibition observed by RINKKIEK (data not shown). Clearly not all proteins interfere with the LOX-RINKKIEK interaction, and the extent of this interference may be governed by the nature and concentration of the target protein. This is an important observation, because any interaction of RINKKIEK (or other potential inhibitor)
with other proteins may hamper the application of this type of inhibitors in complex matrices such as food.

**LOX-1 binding versus LOX-1 inhibition and 15-LOX inhibition**

The results of the present study show that there is a clear correlation between LOX-1 binding and LOX-1 inhibition (Tables 4.1 and 4.2). Strong binding usually results in a low LOX-1 activity and therefore the peptide libraries prepared by SPOT synthesis are suitable for screening and optimization of potential LOX inhibitors. RINKKIEK and some analogs are also inhibitors of mammalian 15-LOX. However, there is no correlation between the results obtained with soybean LOX-1 and rabbit 15-LOX. This is not unexpected considering the low sequence identity between plant and mammalian LOXs of 21-27% (Prigge, 1996). The sequence identity is the highest in the catalytic domain near the iron-binding site. Since LOX-1 inhibition by peptides is mainly noncompetitive, binding of the peptides is likely to occur outside the active site in the non-conserved regions. The mechanism of inhibition of 15-LOX by these peptides may be quite different and requires further investigation.

**Conclusions**

Within RINKKIEK, the positively charged amino acid residues are very important for the LOX-RINKKIEK interaction. A positive charge promotes the interaction, whereas a negative charge diminishes the interaction. The LOX-RINKKIEK binding is significantly improved upon replacement of the negatively charged Glu. RINKKIPK and RINKKISK show a significant higher affinity for LOX-1 than RINKKIEK and a threefold stronger, noncompetitive LOX-1 inhibition. RINKKIEK and some analogs are inhibitors of mammalian 15-LOX as well. The specificity of the LOX-RINKKIEK interaction appears to be low. Although a significant improvement of LOX-1 inhibition by peptides was achieved, there is still the need to investigate the applicability of these peptides in food and/or therapeutics.

**Acknowledgements**

This work was supported by the Ministry of Economic Affairs of The Netherlands, the innovation driven program IOP Industrial Proteins (project IIE00022) and by Cargill, Cebeco Egg Research, Cosmoferm BV and DMV International. We thank W.A.M. van den Berg for his support in the tryptophan fluorescence measurements.
Lipoxygenase inhibitory peptides
Part II

Tyrosinase inhibition
Chapter 5

Inhibition of polyphenol oxidases by proteins and peptides
Polyphenol oxidases: occurrence, mechanism and function

PPOs are a family of type 3 copper-containing proteins, present in animals, plants, fungi, and microorganisms, which catalyze the oxidation of monophenols to diphenols and/or the oxidation of diphenols further to reactive quinones in the presence of oxygen (Halaouli, 2006; Lind, 1999; Mayer, 1979; Mayer, 2006; Yoruk, 2003b). Different types of PPOs exist: catechol oxidases (EC 1.10.3.1) catalyze the oxidation of o-diphenols to o-quinones, laccases (EC 1.10.3.2) catalyze the oxidation of o- and p-diphenols to o- and p-quinones, and tyrosinases (EC 1.14.18.1) catalyze the o-hydroxylation of monophenols (cresolase or monophenolase activity) and the subsequent oxidation of the resulting o-diphenols into reactive o-quinones (catecholase or diphenolase activity) (Figure 5.1). Both tyrosinase (TYR) activities appear to have broad substrate specificities, although the enzyme has a higher affinity for the L-isomers of the substrates than for the corresponding D-isomers (Espín, 1998a). TYRs are involved in the melanin pathway and are especially responsible for the first steps of melanin synthesis leading to the formation of L-DOPAquinone and L-DOPAchrome (Sánchez-Ferrer, 1995). The reactive quinones formed are involved in a cascade of oxidative condensation and addition reactions with phenolic compounds, thiols and primary or secondary amines either free or present in proteins, leading to the formation of melanin. Melanins are heterogeneous polyphenol-like biopolymers with a complex structure and color varying from yellow to black (Figure 5.3). Their biosynthesis can be observed by anyone who leaves the surface of a cut apple, potato or banana exposed to air.

Figure 5.1 – Proposed catalytic mechanism of TYR. TYR catalyzes the o-hydroxylation of monophenols and the subsequent conversion of the catechols to the corresponding o-quinones (Matoba, 2006).
Quaternary structure of mushroom tyrosinase

Fungal TYRs were firstly characterized from the edible mushroom *Agaricus bisporus* (Jolley, 1969; Long, 1969; Nakamura, 1966). TYRs are cytosolic enzymes, which have considerable heterogeneity compared to other copper-containing PPOs such as, for example, laccases. Several TYR forms with different molecular masses have been isolated. It has been proposed that mushroom TYR consists of two different subunits, a heavy (H) chain and a light (L) chain with sizes of 43 and 13.4 kD, respectively (Strothkamp, 1976). In aqueous solution the predominant form of TYR has the quaternary structure \((HL)_2\) with an estimated molecular mass of 120 kD. The heterotetrameric structure of *Agaricus bisporus* TYR is rather unusual, since other PPOs from bacteria, fungi, higher plants or animals contain only one subunit type. Two types of H chains have been identified (Robb, 1981), thus polymorphism of TYR could be attributed to conformational isomerism, differences in genetic expression, or various post-translational modifications of a single primary gene product, for example limited proteolysis or glycosylation. Based on isoelectric point, two groups of *Agaricus bisporus* TYRs were isolated, one group with pI between 4-4.5 and the other with pI between 4.5-5 (Flurkey, 1991). Later it was shown that the lower-pI TYRs were glycosylated whereas the higher-pI TYRs were not (Gerritsen, 1994). Two monomeric TYRs, PPO1 and PPO2, were isolated from fruitbodies of the *Agaricus bisporus* strain U1 (Wichers, 1996). Both TYRs, with a pI around 5.2 and 5.1, showed a molecular mass of about 43 kD and were fully active. The two TYR isoforms are encoded by two different genes (Wichers, 2003). The ORFs for PPO1 and PPO2 were shown to encode TYRs of about 64 kD. TYRs were shown to exist in both latent (inactive) and active forms with the monomeric form of about 64 kD being the latent form. In *Agaricus bisporus* the latent form represented about 97-99% of the total TYR pool (van Leeuwen, 1999). *In vitro*, latent fungal TYRs could be activated by acidic shocks (Ichishima, 1984), serine proteases (Espín, 1999a), and sodium dodecyl sulphate (Espín, 1999b). *In vivo*, TYR activation was assumed to be the result of ageing (Burton, 1993), exposure to extreme environmental conditions or to pathogens (Jolivet, 1998; Soler-Rivas, 2001). The mechanism of fungal activation is still unclear, but it is supposed to be the consequence of endogenous proteases and/or conformational changes (Espín, 2000a). Activation of the latent TYR by proteases yields a 58 kD and a fully active active 43 kD fragment corresponding to the H chain. Therefore, fragments of about 6 kD and of about 15 kD are hypothetically split off during the proteolytic activation process. Assuming that the latent TYR present in mushrooms becomes activated during the isolation procedure, it is possible that the smaller fragment observed in mushroom TYR preparations represents the L chain based on similar size.
Crystal structures of type 3 copper proteins

All TYRs examined so far comprise two rather well conserved regions generally termed CuA and CuB, which contain strictly conserved amino acid residues, including three histidines (García-Borron, 2002; van Gelder, 1997; Marusek, 2006). These regions participate in the structure of the active site as copper-binding molecules. Each atom of the binuclear copper cluster is ligated to three histidines. Crystal structures of type 3 copper proteins currently known are *Limulus polyphemus* hemocyanin (Magnus, 1994), *Octopus dofleini* hemocyanin (Cuff, 1998), *Ipomoea batatas* catechol oxidase (Klabunde, 1998), and the recently solved structure of TYR from *Streptomyces castaneoglobisporus* (Matoba, 2006). A superposition of these four proteins shows a high degree of structural similarity of the active sites (Decker, 2006). Although the active centers of the type 3 proteins are similar in both their overall structure and their ability to bind molecular oxygen, their functions are quite different. Based on the crystal structures, the difference in catalytic activity between catechol oxidase and TYR may be explained by a large vacant space just above the active center of TYR, whereas in catechol oxidase the CuA site is shielded by Phe261 (Decker, 2000b; Eicken, 1999). Orientation of phenols with their hydroxyl group pointing toward CuA appears to be necessary for the hydroxylation reaction. Bonding of diphenols is assumed to occur to CuB (Decker, 2000a).

![Figure 5.2 – Structures of different type 3 copper proteins with ribbon presentation of their inhibitory domains (Decker, 2007).](image)

(A) *Limulus polyphemus* hemocyanin, (B) *Octopus dofleini* hemocyanin, (C) *Ipomoea batatas* catechol oxidase, (D) *Streptomyces castaneoglobisporus* tyrosinase.

The crystal structure of TYR from *Streptomyces castaneoglobisporus* was solved in complex with ORF378 to facilitate crystallization (Matoba, 2006). ORF378 is referred to as a caddie protein, because it assists with transportation of cupric ions to the active site of TYR during maturation. This caddie protein covers the hydrophobic active site of TYR with its Tyr98 residue present in the substrate-binding pocket (Decker, 2007). This blocking residue is not converted to DOPA and it is suggested that ORF378 functions as an inhibitor of TYR (Figure
5.2). This inhibitor is comparable to the inhibitory domains observed in other type 3 copper proteins. Enzyme activation by dissociation of the covering domain also involves removal of the blocking residue, allowing substrates to interact with the active site.

**Biological role of tyrosinases**

TYRs are mainly associated with browning and pigmentation. Melanins are formed by a combination of enzymatically catalyzed and chemical reactions (Figure 5.3). The melanin plays an important role in protecting human skin from the harmful effects of UV radiation from the sun and in scavenging toxic drugs and chemicals. It determines our phenotypic appearance. Furthermore, melanin pigments are also found in the mammalian brain. TYR plays a role in neuromelanin and DOPAmine formation in the human brain (Rodgers, 1975). The color of mammalian skin and hair is determined by a number of factors, the most important of which is the degree and distribution of melanin pigmentation. Upon exposure of the skin to UV radiation (Nylander, 2000), melanogenesis is initiated with the first step of tyrosine hydroxylation catalyzed by TYR. This first step is the rate-limiting step in melanin synthesis and defects in the enzyme’s activity leads to albinism in humans (Goding, 2007; Oetting, 1999). Melanin is formed in specialized pigment-producing, dendritic cells known as melanocytes (Westerhof, 2006). The migration of melanocytes throughout an organism during development is under strict genetic control (Sturm, 2001) and leads to some interesting results if their final distribution in the skin is not uniform. The characteristic skin patterns of, for example, zebras or giraffes are due to this uneven distribution of melanocytes. Vitiligo is an autoimmune disorder in humans, whereby patches of hypopigmented skin result from loss of melanocytes (Spritz, 2006). At a cellular level, melanin pigments are biosynthesized in the membranous organelles named melanosomes. Melanosomes contain about 1500 proteins of which approximately 100 define the essential melanosome proteome (Chi, 2006). Eumelanosomes produce dark melanins (Meredith, 2006), whereas the pheomelanosomes produce light red/yellowish melanins (Napolitano, 1996) (Figure 5.3). The availability of the sulfhydryl compounds leads to the synthesis of pheomelanin. The thiol group of cysteine (or glutathione) can add to different ring positions, although the 5-position is favored. The mature melanosomes located in the dendrites of melanocytes are phagocytosed by the surrounding recipient cells, keratinocytes, and it is this process that is responsible for the variety of colors in human skin, hair and eyes. The type and amount of melanin synthesized by the melanocyte, and its distribution pattern in the surrounding keratinocytes, determines the actual color.
Figure 5.3 - Raper-Mason pathway of melangogenesis in mammals (del Marmol, 1996; Sánchez-Ferrer, 1995; Seo, 2003). TYR, tyrosinase; TRP, tyrosinase related protein; DCT, DOPAchrome tautomerase; DOPA, 3,4-dihydroxyphenylalanine; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DHI, 5,6-dihydroxyindole; ICAQ, indole-2-carboxylic acid-5,6-quinone; IQ, indole-5,6-quinone; HBTA, 5-hydroxy-1,4-benzothiazylalanine; DHCHBTA, 2-dihydro-3-carboxy-5-hydroxy-1,4-benzothiazyl-alanine.
In fungi, melanins may constitute a mechanism of defence and resistance to stress such as UV radiations, free radicals, dehydration and extreme temperatures, and contribute to the fungal cell wall resistance against hydrolytic enzymes in avoiding cellular lysis. Furthermore, fungal pigments are also involved in the formation and stability of spores (Mayer, 1979) and possibly in defence and virulence mechanisms (Jacobson, 2000; Soler-Rivas, 1997).

Implications of melanin formation in food and health

**Negative effects of tyrosinase activity on health**

Although the melanin has mainly a (photo)protective function in the human skin, the process of melanogenesis represents a potential cellular hazard and is therefore confined to melanosomes, specialized organelles located within melanocytes. In some cases, for example due to excessive sun exposure, melanocytes exhibit up-regulated melanogenesis and defective melanosomes. When the biosynthesis of the pigment is released to the cytosol, the reactive quinone intermediates may cause oxidative damage and interfere with other cellular processes. As electrophiles, quinones can form covalent adducts with cellular macromolecules, including DNA. The damaged DNA may result in mutations leading to different types of cancer (Cavalieri, 2002). Macromolecular damage, combined with increased oxidative stress, may trigger cellular responses that eventually lead to cell death. Hence, the reactive quinones generated by TYR in the brain may also play a role in neurodegenerative processes like Parkinson’s disease (Asanuma, 2003; Stokes, 1999; Tief, 1998). Besides the pathological processes associated with TYR activity, the accumulation of an abnormal melanin amount in different specific parts of the skin resulting in more pigmented patches might become an esthetic problem. In western countries, skin lighteners are applied for the prevention and treatment of irregular hyperpigmentation, such as freckles, melasma, or age spots. TYR inhibition is the most common approach to achieve skin depigmentation as this enzyme catalyzes the rate-limiting step of melanin formation (Briganti, 2003; Parvez, 2006; Solano, 2006).

**Negative effects of tyrosinase activity in food**

PPOs are responsible for browning that occurs in many natural food products. Enzymatic browning is beneficial for developing color, flavour and aroma in various products as cacao, tea, coffee, wine, tobacco, and dried fruits such as figs and raisins (Bittner, 2006). However, this browning process is undesirable in for example fresh fruits, beverages, vegetables, and...
mushrooms (Artés, 1998; Rescigno, 2002). Browning after harvest is a common phenomenon in crops as mushrooms, which decreases the commercial value of the products. Loss of whiteness upon storage is a big issue in the mushroom industry (Jolivet, 1998; Rajaratnam, 2003). Bruising, fruiting body senescence and bacterial infection initiate discoloration reactions. Explanations for these complex processes are rupture of cell structure and the accompanied decompartmentation between substrates and enzymes following breakdown of intracellular membranes and the post-harvest activation of latent PPOs by proteases (Espín, 1999a). Diffusing polyphenols are then oxidized by PPOs and the resulting quinones associate with proteins, peptides and amino acids (Bittner, 2006). The quinone-protein reaction decreases the digestibility of the protein and the bioavailability of essential amino acids, including lysine and cysteine. Enzymatic browning in foods is usually controlled with chemicals, heat treatment, or low oxygen atmosphere. Lemon juice and other acids are used to preserve color in fruits and juices by lowering the pH. Especially sulfites are commonly used to control browning. However, negative health effects associated with sulfites have created the need for functional alternatives (Iyengar, 1992; McEvily, 1992).

**Common tyrosinase inhibitors**

Inhibition of melanin formation is an important target in fields of medicine, cosmetic industry, and agriculture (Kim, 2005a). Antibrowning agents can act at different levels in the melanogenesis pathway of which a direct inhibition of the initiating PPO is most effective. Inhibition of PPO activity can be accomplished by reducing agents causing chemical reduction of pigment precursors, acidulants such as citric acid to lower the pH below minimum pH required for catalytic activity, chelating agents that can either bind to the copper in the active site or reduce the level of copper available for incorporation into the holoenzyme, or specific inhibitors of PPO that are, for example, acting upon the substrate binding site. TYR from mushroom is the most widely studied PPO and a number of inhibitors from both natural and synthetic sources that inhibited monophenolase, diphenolase or both of these activities have been identified to date (Table 5.1). The $IC_{50}$ values reported in literature depend on the assay conditions applied. In order to compare the potencies of the various inhibitors, only $IC_{50}$ values determined using the natural substrates of mushroom TYR are shown. Recently, a more extended overview of 245 TYR inhibitors and their potencies was published as part of a QSAR study to identify new TYR inhibitory compounds (Casañola-Martín, 2007).
Table 5.1 – Overview of some common inhibitors of mushroom TYR and reported \( IC_{50} \) values.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( IC_{50} / \mu M )</th>
<th>Type of inhibition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agaritine</td>
<td>n.d. [a]</td>
<td>competitive</td>
<td>(Espín, 1998b)</td>
</tr>
<tr>
<td></td>
<td>3000 [b]</td>
<td>uncompetitive</td>
<td>(Espín, 1998b)</td>
</tr>
<tr>
<td>Aloesin</td>
<td>100 [a]</td>
<td>noncompetitive</td>
<td>(Jin, 1999)</td>
</tr>
<tr>
<td>Anisaldehyde</td>
<td>320 [b]</td>
<td>noncompetitive</td>
<td>(Kubo, 1998a)</td>
</tr>
<tr>
<td></td>
<td>380 [b]</td>
<td>noncompetitive</td>
<td>(Lee, 2002)</td>
</tr>
<tr>
<td></td>
<td>400 [b]</td>
<td>noncompetitive</td>
<td>(Kubo, 2003)</td>
</tr>
<tr>
<td>Anisic acid</td>
<td>640 [b]</td>
<td>noncompetitive</td>
<td>(Kubo, 1998b)</td>
</tr>
<tr>
<td></td>
<td>680 [b]</td>
<td>noncompetitive</td>
<td>(Lee, 2002)</td>
</tr>
<tr>
<td></td>
<td>600 [b]</td>
<td>noncompetitive</td>
<td>(Kubo, 2003)</td>
</tr>
<tr>
<td>Arbutin</td>
<td>5000</td>
<td>n.d.</td>
<td>(Kawagishi, 1993)</td>
</tr>
<tr>
<td></td>
<td>40 [a]</td>
<td>competitive</td>
<td>(Jin, 1999)</td>
</tr>
<tr>
<td></td>
<td>55 [a]</td>
<td>n.d.</td>
<td>(Ha, 2001)</td>
</tr>
<tr>
<td></td>
<td>1113 [b]</td>
<td>n.d.</td>
<td>(Jones, 2002)</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>284 [b]</td>
<td>n.d.</td>
<td>(Wu, 2003b)</td>
</tr>
<tr>
<td></td>
<td>30 [b]</td>
<td>n.d.</td>
<td>(Um, 2003)</td>
</tr>
<tr>
<td>Baicalein</td>
<td>290 [b]</td>
<td>n.d.</td>
<td>(Kubo, 2000a)</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>820 [b]</td>
<td>noncompetitive</td>
<td>(Kubo, 1998a; Kubo, 1998b)</td>
</tr>
<tr>
<td></td>
<td>830 [b]</td>
<td>noncompetitive</td>
<td>(Lee, 2002)</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>640 [b]</td>
<td>mixed</td>
<td>(Kubo, 1998b)</td>
</tr>
<tr>
<td></td>
<td>710 [b]</td>
<td>competitive</td>
<td>(Lee, 2002)</td>
</tr>
<tr>
<td>Captopril</td>
<td>n.d. [a]</td>
<td>noncompetitive</td>
<td>(Espin, 2001)</td>
</tr>
<tr>
<td></td>
<td>150 [b]</td>
<td>competitive</td>
<td>(Espin, 2001)</td>
</tr>
<tr>
<td>Inhibitor</td>
<td>$IC_{50}$ / $\mu M$</td>
<td>Type of inhibition</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>---------------------</td>
<td>--------------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>Chamaecin</td>
<td>2.3$^{[b]}$</td>
<td>mixed</td>
<td>(Nihei, 2004)</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>980$^{[b]}$</td>
<td>noncompetitive</td>
<td>(Kubo, 1999c; Lim, 1999)</td>
</tr>
<tr>
<td></td>
<td>970$^{[b]}$</td>
<td>noncompetitive</td>
<td>(Lee, 2002)</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>720$^{[b]}$</td>
<td>mixed</td>
<td>(Lim, 1999)</td>
</tr>
<tr>
<td></td>
<td>700$^{[b]}$</td>
<td>mixed</td>
<td>(Lee, 2002)</td>
</tr>
<tr>
<td></td>
<td>2100$^{[b]}$</td>
<td>noncompetitive</td>
<td>(Shi, 2005)</td>
</tr>
<tr>
<td>$p$-Coumaric acid</td>
<td>3650$^{[b]}$</td>
<td>mixed</td>
<td>(Lim, 1999)</td>
</tr>
<tr>
<td>Cumic acid</td>
<td>260$^{[b]}$</td>
<td>noncompetitive</td>
<td>(Kubo, 1998b)</td>
</tr>
<tr>
<td>Cuminaldehyde</td>
<td>50$^{[b]}$</td>
<td>noncompetitive</td>
<td>(Kubo, 1998b)</td>
</tr>
<tr>
<td></td>
<td>170$^{[a]}$</td>
<td>competitive</td>
<td>(Jiménez, 2001)</td>
</tr>
<tr>
<td></td>
<td>40$^{[b]}$</td>
<td>competitive</td>
<td>(Jiménez, 2001)</td>
</tr>
<tr>
<td>Cupferron</td>
<td>1.1$^{[b]}$</td>
<td>competitive</td>
<td>(Shiino, 2001)</td>
</tr>
<tr>
<td></td>
<td>0.52$^{[a]}$</td>
<td>competitive</td>
<td>(Xie, 2003a)</td>
</tr>
<tr>
<td></td>
<td>0.84$^{[b]}$</td>
<td>competitive</td>
<td>(Xie, 2003a)</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>310$^{[b]}$</td>
<td>n.d.</td>
<td>(Kahn, 1985a)</td>
</tr>
<tr>
<td></td>
<td>350$^{[a]}$</td>
<td>n.d.</td>
<td>(Friedman, 1995)</td>
</tr>
<tr>
<td>4,4’-Dihydroxybiphenyl</td>
<td>1.91$^{[a]}$</td>
<td>competitive</td>
<td>(Kim, 2005b)</td>
</tr>
<tr>
<td>trans-3,5-Dihydroxystilbene</td>
<td>705$^{[b]}$</td>
<td>competitive</td>
<td>(Song, 2006)</td>
</tr>
<tr>
<td>Esculetin</td>
<td>43$^{[b]}$</td>
<td>competitive</td>
<td>(Masamoto, 2003)</td>
</tr>
<tr>
<td>L-Glutathione</td>
<td>120$^{[a]}$</td>
<td>n.d.</td>
<td>(Friedman, 1995)</td>
</tr>
</tbody>
</table>
Table 5.1 (continued) – Overview of some common inhibitors of mushroom TYR and reported \( IC_{50} \) values.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( IC_{50} / \mu M )</th>
<th>Type of inhibition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Hydroxyanisole</td>
<td>150(^{[b]})</td>
<td>n.d.</td>
<td>(Kubo, 1998a)</td>
</tr>
<tr>
<td>p-Hydroxybenzaldehyde</td>
<td>1200(^{[b]})</td>
<td>competitive</td>
<td>(Kubo, 1998a)</td>
</tr>
<tr>
<td>4-Hydroxychalcone</td>
<td>21.8(^{[a]})</td>
<td>competitive</td>
<td>(Nerya, 2004)</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>75(^{[a]})</td>
<td>competitive</td>
<td>(Briganti, 2003)</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>230(^{[b]})</td>
<td>competitive</td>
<td>(Kubo, 1999b; Kubo, 2000a)</td>
</tr>
<tr>
<td></td>
<td>3.49(^{[a]})</td>
<td>n.d.</td>
<td>(Kim, 2006)</td>
</tr>
<tr>
<td></td>
<td>42(^{[a]})</td>
<td>competitive</td>
<td>(Lim, 2006)</td>
</tr>
<tr>
<td></td>
<td>110(^{[a]})</td>
<td>competitive</td>
<td>(Chen, 1991b)</td>
</tr>
<tr>
<td></td>
<td>98(^{[b]})</td>
<td>mixed</td>
<td>(Chen, 1991b)</td>
</tr>
<tr>
<td></td>
<td>120(^{[b]})</td>
<td>n.d.</td>
<td>(Maeda, 1996)</td>
</tr>
<tr>
<td></td>
<td>99(^{[a]})</td>
<td>n.d.</td>
<td>(Ha, 2001)</td>
</tr>
<tr>
<td></td>
<td>40.1(^{[a]})</td>
<td>competitive</td>
<td>(Kim, 2002)</td>
</tr>
<tr>
<td></td>
<td>90(^{[a]})</td>
<td>n.d.</td>
<td>(Piao, 2002)</td>
</tr>
<tr>
<td></td>
<td>10(^{[b]})</td>
<td>n.d.</td>
<td>(Jones, 2002)</td>
</tr>
<tr>
<td></td>
<td>77.4(^{[b]})</td>
<td>n.d.</td>
<td>(Sasaki, 2002)</td>
</tr>
<tr>
<td></td>
<td>35(^{[b]})</td>
<td>n.d.</td>
<td>(Wu, 2003b)</td>
</tr>
<tr>
<td></td>
<td>60(^{[b]})</td>
<td>n.d.</td>
<td>(Um, 2003)</td>
</tr>
<tr>
<td></td>
<td>10(^{[b]})</td>
<td>mixed</td>
<td>(Masamoto, 2003)</td>
</tr>
<tr>
<td></td>
<td>22.94(^{[a]})</td>
<td>n.d.</td>
<td>(Kim, 2004)</td>
</tr>
<tr>
<td></td>
<td>16.3(^{[b]})</td>
<td>n.d.</td>
<td>(Cho, 2006)</td>
</tr>
<tr>
<td></td>
<td>16.67(^{[b]})</td>
<td>n.d.</td>
<td>(Khan, 2006a; Khan, 2006b)</td>
</tr>
<tr>
<td></td>
<td>4.0(^{[b]})</td>
<td>noncompetitive</td>
<td>(Li, 2006)</td>
</tr>
<tr>
<td>L-Mimosine</td>
<td>160(^{[b]})</td>
<td>n.d.</td>
<td>(Andrawis, 1985)</td>
</tr>
<tr>
<td></td>
<td>340(^{[b]})</td>
<td>competitive</td>
<td>(Cabanes, 1987; Parvez, 2006)</td>
</tr>
<tr>
<td></td>
<td>3.68(^{[b]})</td>
<td>n.d.</td>
<td>(Khan, 2006a; Khan, 2006b)</td>
</tr>
<tr>
<td>Oxyresveratrol</td>
<td>1(^{[b]})</td>
<td>noncompetitive</td>
<td>(Shin, 1998)</td>
</tr>
<tr>
<td></td>
<td>1.2(^{[a]})</td>
<td>noncompetitive</td>
<td>(Kim, 2002)</td>
</tr>
<tr>
<td>Propyl gallate</td>
<td>310(^{[b]})</td>
<td>n.d.</td>
<td>(Kubo, 1999a; Kubo, 2000b)</td>
</tr>
</tbody>
</table>
Table 5.1 (continued) – Overview of some common inhibitors of mushroom TYR and reported $IC_{50}$ values.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$IC_{50}$ / µM</th>
<th>Type of inhibition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3COH3CO</td>
<td>20[a]</td>
<td>competitive</td>
<td>(No, 2004)</td>
</tr>
<tr>
<td>Protocatechuic aldehyde</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOCOC6O</td>
<td>20[a]</td>
<td>competitive</td>
<td>(Kubo, 1999b; Kubo, 2000a)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>130[b]</td>
<td>competitive</td>
<td>(Chen, 2002)</td>
</tr>
<tr>
<td></td>
<td>50[b]</td>
<td>competitive</td>
<td>(Xie, 2003b)</td>
</tr>
<tr>
<td>OHOH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OHOH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resveratrol</td>
<td>54.6[a]</td>
<td>n.d.</td>
<td>(Kim, 2002)</td>
</tr>
<tr>
<td>OHOH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>4300[b]</td>
<td>competitive</td>
<td>(Zhang, 2006)</td>
</tr>
<tr>
<td>OHOH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tropolone</td>
<td>2.1[a]</td>
<td>n.d.</td>
<td>(Kahn, 1985b)</td>
</tr>
<tr>
<td></td>
<td>0.4[b]</td>
<td>mixed</td>
<td>(Kahn, 1985b)</td>
</tr>
<tr>
<td>OHOH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanillin</td>
<td>70000[b]</td>
<td>n.d.</td>
<td>(Kubo, 1999b)</td>
</tr>
</tbody>
</table>

[a] With L-Tyr as a substrate; [b] With D- and/or L-DOPA as a substrate.

The most intensively studied inhibitor of TYR is kojic acid, a fungal metabolite currently applied as a cosmetic skin-whitening agent and as a food additive for preventing enzymatic browning (Burdock, 2001). Kojic acid shows a competitive inhibitory effect on the monophenolase activity and a mixed inhibitory effect on the diphenolase activity of mushroom TYR (Table 5.1). The ability of kojic acid to chelate copper at the active site of the TYR enzyme, may well explain the observed competitive inhibitory effect (Battaini, 2000). Besides this, it is reported that the quinone formed by TYR oxidizes kojic acid to form a yellow product (Chen, 1991a; Kahn, 1995; Kahn, 1997). Therefore, kojic acid also acts as a chemical antioxidant reducing the quinone formed and this way inhibits melanin formation. Kojic acid is reported to be a slow-binding inhibitor of diphenolase activity of TYR (Cabanes, 1994). This means that the active form of TYR, generated in the catalytic cycle in presence of substrate, is required before binding of the inhibitor to the enzyme can occur. Other slow-
binding inhibitors of TYR are the very potent inhibitor tropolone (Espín, 1999c) and a substrate analog L-mimosine (Cabanes, 1987). Strikingly, these slow-binding inhibitors of TYR all contain a $\alpha$-hydroxyketo group.

As with most LOX inhibitors (Table 2.1), many common TYR inhibitors are also phenolic compounds. Since several polyphenols are accepted as substrates by PPOs, it depends on the presence and position of additional substituents whether a phenolic compound may act as an inhibitor (Kanade, 2007). Polyphenols represent a diverse group of compounds containing multiple phenolic functionalities and are widely distributed in nature. Flavonoids are among the most numerous and best-studied polyphenols. Some potent TYR inhibitory flavonoids, such as quercetin and kaempferol, have been isolated from various plants. A number of studies have been carried out in order to determine the relationship between the TYR inhibitory activity of flavonoids and their structure (Kubo, 2000a; Shimizu, 2000). The inhibitory ability may be explained in terms of similarity between the catechol moiety in L-DOPA and the dihydroxy group(s) (Kim, 2006) or between kojic acid and the $\alpha$-hydroxyketo group (Kubo, 1999b; Xie, 2003b) in flavonoids. Quercetin and kaempferol competitively inhibit TYR by their ability to chelate the copper in the active site, leading to inactivation of TYR. The importance of phenolic hydroxyl groups in TYR inhibition is also demonstrated by a group of stilbene compounds. Oxyresveratrol appears to be a very potent, noncompetitive inhibitor of both the monophenolase and diphenolase activity of mushroom TYR. This inhibitory effect is decreased upon removal of one (resveratrol) or more hydroxyl groups (Song, 2006), or by replacement of these hydroxyl groups with methoxy groups (Kim, 2002). A study testing various chalcones as inhibitors of TYR (Nerya, 2004), demonstrated that the position of hydroxyl groups on the aromatic ring is more important than the total number of phenolic hydroxyl groups present within the molecule. Another group of polyphenols studied are the gallate esters. In contrast to LOX inhibition, the length of the hydrophobic chain in gallate esters does not affect TYR inhibition (Kubo, 2000b). However, the short alkyl chain ($<C_{10}$) gallate esters were oxidized themselves by TYR, so the long alkyl chain ($>C_{10}$) gallate esters are more useful as TYR inhibitors. As applies for most polyphenols, the inhibitory effect of gallate esters most likely results from scavenging the radicals involved in melanin formation. Other TYR inhibitory compounds include benzoic acid and benzaldehyde derivatives. It is proposed that the TYR inhibitory potency of several aldehydes (Table 5.2) presumably results from their ability to form a Schiff base with a primary amino group in the enzyme (Kubo, 1998b). Although anisaldehyde is reported to be an inhibitor of TYR, it rather activates melanogenesis in melanoma cells (Nitoda, 2007).
Tyrosinase inhibitory peptides

Only a few of the antibrowning agents listed above (Table 5.2), typically kojic acid, are currently commercially available. They provide some cause for concern, for example high toxicity toward cells, and low stability toward oxygen and water, resulting in their limited application. Safety is a primary consideration for TYR inhibitors, especially those in food and cosmetic products (Seo, 2003). Hence, inhibitors such as peptides from natural sources have great potential in controlling browning of foodstuffs and preventing hyperpigmentation disorders and other TYR-related health problems in humans.

Proteins and peptides as inhibitors of melanin formation

Proteins, peptides and amino acids can affect TYR activity either through direct inhibition of the enzyme or by reacting with the quinone products of TYR activity (McEvily, 1992). N-terminal primary amino groups, aliphatic amino groups (secondary amines in amino acids) and thiol-containing amino acids react with \(\alpha\)-benzoquinones and 4-methyl-\(\alpha\)-benzoquinone, while only thiol-containing compounds (and aromatic amines) react with oxidation products of \(\alpha\)-L-DOPA (Friedman, 1995; Mason, 1965). Interaction of \(\alpha\)-quinones with certain amino acids may result in a substantial change in the level of colored melanins that can otherwise be formed by the action of TYR on phenolic substrates. In addition, proteins and peptides are also capable of chelating the essential copper at the active site of TYR, theoretically resulting in the inhibition of TYR activity.

Proteins and peptides from milk and egg

The effects of bovine milk proteins on melanogenesis were examined. Both whey protein isolate and casein exhibited depigmenting properties when added to cultured melanocytes (Nakajima, 1996). Among the major protein components of milk, including \(\beta\)-lactoglobulin, \(\alpha\)-lactalbumin, \(\alpha\)-, \(\beta\)-, and \(\kappa\)-casein, only \(\kappa\)-casein exhibited the depigmenting activity. However, the carboxyl terminal peptide of \(\kappa\)-casein did not show this effect. Since \(\kappa\)-casein promoted the proliferation of the cells, the observed depigmenting effect could not be attributed to a decrease in cell number. The results suggest that the melanogenesis-suppressing activity of \(\kappa\)-casein is mediated by inhibition of TYR activity (Nakajima, 1996). Other milk proteins were proposed to inhibit TYR as well. The use of lactoferrin hydrolyzate was claimed as a TYR inhibition agent (Tomita, 1995). Another patent application describes the use of enzymatic hydrolyzates of milk proteins for inhibiting TYR activity in treatment of skin (Tomita, 1993). Both kefir whey and kefir whey peptides could chelate the copper in
TYR, which might explain the mechanism of inhibition (Chen, 2006). Lysozyme from egg white is claimed to be a 10-fold better inhibitor of mushroom TYR than kojic acid (Li, 2006). However, taken into account the high molecular mass of lysozyme compared to kojic acid, kojic acid is actually 3 times better than lysozyme on a gram scale. Nevertheless, lysozyme is able to inhibit TYR at low concentrations.

![Sequence and location of disulfide bridges within human insulin (A) and the phenoloxidase inhibitor (POI) protein from the housefly (B). The tyrosine residue at the C-terminal end of the POI peptide may be posttranslationally oxidized to L-DOPA.](image)

**Protein inhibitors of human origin**

Some peptides present in the human body are reported to possess TYR inhibiting activity. A 66 kD protein from human cytosol inhibits human skin TYR competitively with tyrosine (Vijayan, 1982). In another study, a single polypeptide protein purified from melanosomes of normal skin epidermal melanocytes with the same molecular mass partially inhibits human skin TYR (Babu, 1998). Although the identity of the protein in both studies is unknown, it may well be human serum albumin (HSA) considering its abundance and molecular mass. Insulin, another protein of human origin, is proposed to be an inhibitor of TYR as well. Insulin (Figure 5.4A) inhibits the formation of melanin via the inhibition of TYR activity (Benathan, 1997).

**Melanization inhibitors from arthropods**

The melanization reaction induced by phenoloxidase in arthropods is important in the multiple host defense reactions. This reaction ought to be tightly controlled because excessive formation of quinones resulting in hypermelanization is deleterious to the host, suggesting that a negative regulator of melanin synthesis may exist in hemolymph. A phenoloxidase inhibitor (POI) from the hemolymph of the housefly *Musca domestica* had been determined to competitively inhibit phenoloxidase with a $K_i$ around 10 nM (Daquinag, 1995; Daquinag, 1999; Tsukamoto, 1992). POI is a cysteine-rich protein containing three disulfide bridges (Figure 5.4B). Later this protein appeared to be able to inhibit an apple PPO
as well (Yoruk, 2003a). Another melanization-inhibiting protein (MIP) was cloned from the mealworm *Tenebrio molitor* (Zhao, 2005). Although the amino acid sequence of this 43 kD protein did not show any homology with that of POI, the results suggest that this protein inhibits the formation of melanin. MIP may function as a scavenger-like compound against reactive quinone products. Furthermore, silk protein sercin from the silk worm *Bombyx mori* not only suppressed *in vitro* lipid oxidation, but was also found to inhibit TYR activity. Because of this dual effect it may be a valuable ingredient for food and cosmetics (Kato, 1998; Mase, 2006).

**Honey**

In general, the antioxidant capacity of honey appears to be a result of the combined activity of a wide range of compounds including phenolics, peptides, organic acids, enzymes, Maillard reaction products, and possibly other minor components. The phenolic compounds contribute significantly to the antioxidant capacity of honey, but are not solely responsible for it (Chen, 2000; Gheldof, 2002). A small peptide from honey with an approximate molecular weight of 0.6 kD is responsible for a noncompetitive inhibition of TYR (Ates, 2001; Oszmianski, 1990).

**Peptides from fungal origin**

Metallothioneins are small metal-binding proteins thought to play a role in metabolism and detoxification of a number of essential and nonessential trace metals. It was found that metallothioneins, cysteine-rich peptides of low molecular weight involved in subcellular metal storage, can inhibit TYR. Metallothionein from *Aspergillus niger* inhibits the activity of commercially purified mushroom TYR (Goetghebeur, 1996). The mechanism of inhibition of TYR by these low molecular weight peptides is not completely elucidated. Chelation of copper can be a major factor in enzyme inhibition, but other effects, such as direct binding or the reducing properties of the peptides, could result in noncompetitive inhibition. Two other low molecular weight (±1 kD) peptide inhibitors of TYR-mediated DOPA oxidation were isolated from the field mushroom *Agaricus hortensis* (Madhosingh, 1975). One of the two peptidic inhibitors is clearly competitive and contains three major amino acids Phe, Asp and Glu in the approximate ratio 1:1:1 and furthermore contains Ala (Madhosingh, 1974).

**Cyclic peptides**

Several cyclic peptides have been isolated showing potent TYR and melanin-production inhibitory activities (Table 5.2). Pseudostellarins were isolated from the roots of
Pseudostellaria heterophylla and are neutral peptides consisting of five to nine lipophilic amino acids containing proline and aromatic residues (Morita, 1994; Scott, 1999). A cyclotetrapeptide, cyclo(PTPV), showing inhibitory activity against mushroom TYR was isolated from the lactic bacterium Lactobacillus helveticus (Kawagishi, 1993).

**Effect of individual amino acids and small peptides**

The effect of individual amino acids on the activity of mushroom TYR was tested (Kahn, 1985a). It was found that alanine, proline, serine, isoleucine, leucine, asparagine, valine, aspartic acid, glutamic acid and tryptophan had no effect on the \( \alpha \)-dihydroxyphenolase activity of mushroom TYR. In increasing order lysine, glycine, histidine, and phenylalanine inhibited the extent of DOPAchrome production by mushroom TYR from DOPA (Table 5.2). By far the most potent amino acid inhibitor is cysteine, which gives full inhibition of enzymatic browning at low concentrations. Cysteine is proposed to have two effects on the PPO reaction, first reducing the DOPAquinone back to DOPA and second, the most significant effect, conjugate formation with DOPAquinone to prevent melanine formation (Dudley, 1989; Richard-Forget, 1992; Richard, 1991). Cysteine and also glutathione (\( \gamma \)-glutamylcysteinylglycine) prevented the color development by trapping the color intermediates or reducing \( \alpha \)-quinone to colourless compounds (Benjakul, 2006). Glutathione is an ubiquitous compound found in the human body and, among other biological functions, it has been implicated in skin lightening (Villarama, 2005). A dipeptide structurally related to glutathione, \( \gamma \)-EC, formed one thiol-adduct with 4-methylcatechol. This adduct is not a substrate, but rather a competitive inhibitor of endive PPO (Richard-Forget, 1998). Furthermore, other small peptides (di- and triglycine) are also effective inhibitors of mushroom, avocado and banana PPO. In an effort to develop natural and nontoxic inhibitors on the activity of mushroom PPO the effect of various glycyl-dipeptides was investigated (Girelli, 2004). The results show that GD, GF, GG, and GK are mixed-type inhibitors of the enzyme initiated melanin formation, with GD being the most potent inhibitor (Table 5.2). In case of GH, the inhibition is indirect, due to blocking subsequent melanin-forming reactions. For GP and GL, no inhibition was observed.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( IC_{50} / \mu M )</th>
<th>Type of inhibition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>120000[8]</td>
<td>n.d.</td>
<td>(Kahn, 1985a)</td>
</tr>
</tbody>
</table>
## Table 5.2 (continued) – Inhibition of TYR by some natural amino acids, small peptides and proteins.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC$_{50}$ / µM</th>
<th>Type of inhibition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Lysine</td>
<td>160000$^{[b]}$</td>
<td>n.d.</td>
<td>(Kahn, 1985a)</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>65000$^{[b]}$</td>
<td>n.d.</td>
<td>(Kahn, 1985a)</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>53000$^{[b]}$</td>
<td>n.d.</td>
<td>(Andrawis, 1985)</td>
</tr>
<tr>
<td></td>
<td>50000$^{[b]}$</td>
<td>n.d.</td>
<td>(Kahn, 1985a)</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>310$^{[b]}$</td>
<td>n.d.</td>
<td>(Kahn, 1985a)</td>
</tr>
<tr>
<td></td>
<td>500$^{[c]}$</td>
<td>n.d.</td>
<td>(Dudley, 1989)</td>
</tr>
<tr>
<td></td>
<td>350$^{[a]}$</td>
<td>n.d.</td>
<td>(Friedman, 1995)</td>
</tr>
<tr>
<td>L-Homocysteine</td>
<td>230$^{[a]}$</td>
<td>n.d.</td>
<td>(Friedman, 1995)</td>
</tr>
<tr>
<td>L-Cysteinylglycine (CG)</td>
<td>430$^{[a]}$</td>
<td>n.d.</td>
<td>(Friedman, 1995)</td>
</tr>
<tr>
<td>L-Glycylaspartic acid (GD)</td>
<td>$K_i = 660$ µM$^{[b]}$</td>
<td>mixed</td>
<td>(Girelli, 2004)</td>
</tr>
<tr>
<td>L-Glutathione (γ-ECG)</td>
<td>120$^{[a]}$</td>
<td>n.d.</td>
<td>(Friedman, 1995)</td>
</tr>
<tr>
<td>cyclo(PYPV)</td>
<td>1500</td>
<td>n.d.</td>
<td>(Kawagishi, 1993)</td>
</tr>
<tr>
<td>cyclo(GPYLA)</td>
<td>131$^{[a]}$</td>
<td>n.d.</td>
<td>(Morita, 1994)</td>
</tr>
<tr>
<td>cyclo(GIGGPPF)</td>
<td>187$^{[a]}$</td>
<td>n.d.</td>
<td>(Morita, 1994)</td>
</tr>
<tr>
<td>cyclo(GTLSPFLL)</td>
<td>63$^{[a]}$</td>
<td>n.d.</td>
<td>(Morita, 1994)</td>
</tr>
<tr>
<td>cyclo(GGYPLL)</td>
<td>100$^{[a]}$</td>
<td>n.d.</td>
<td>(Morita, 1994)</td>
</tr>
<tr>
<td>cyclo(GPPLGPVF)</td>
<td>175$^{[a]}$</td>
<td>n.d.</td>
<td>(Morita, 1994)</td>
</tr>
<tr>
<td>cyclo(GGYLPPLS)</td>
<td>50$^{[a]}$</td>
<td>n.d.</td>
<td>(Morita, 1994)</td>
</tr>
<tr>
<td>cyclo(PFSFGPLA)</td>
<td>75$^{[a]}$</td>
<td>n.d.</td>
<td>(Morita, 1994)</td>
</tr>
<tr>
<td>4.2 kD POI</td>
<td>5-90 nM$^{[b]}$</td>
<td>competitive (housefly)</td>
<td>(Tsukamoto, 1992)</td>
</tr>
<tr>
<td></td>
<td>11 nM$^{[b]}$</td>
<td>competitive (housefly)</td>
<td>(Daquinag, 1999)</td>
</tr>
<tr>
<td></td>
<td>1.2$^{[c]}$</td>
<td>n.d. (apple)</td>
<td>(Tsukamoto, 1992)</td>
</tr>
<tr>
<td>14 kD Egg white lysozyme</td>
<td>0.32$^{[b]}$</td>
<td>mixed</td>
<td>(Li, 2006)</td>
</tr>
<tr>
<td></td>
<td>0.20$^{[a]}$</td>
<td>mixed</td>
<td>(Li, 2006)</td>
</tr>
<tr>
<td>66 kD Protein (HSA?)</td>
<td>1.3$^{[a]}$</td>
<td>competitive (human)</td>
<td>(Vijayan, 1982)</td>
</tr>
</tbody>
</table>

[a] With L-Tyr as a substrate; [b] With D- and/or L-DOPA as a substrate; [c] With catechol as a substrate.
Derivatives of kojic acid

Amino acid derivatives of kojic acid were synthesized to improve the TYR inhibitory activity. All derivatives showed stronger activities than kojic acid. Among them, the N-kojic-L-phenylalanyl kojiate was the strongest inhibitor and its $IC_{50}$ value was 1/380 that for kojic acid. The inhibition mechanism of these derivatives is similar to that of kojic acid (Kobayashi, 1995; Kobayashi, 1996). A small library of kojic acid tripeptides was prepared by solid-phase parallel synthesis and assayed to evaluate their TYR inhibitory activity (Kim, 2004). Most of the kojic acid tripeptides showed better activities than kojic acid. Kojic acid-FWY was the best compound, and it exhibited 100-fold stronger TYR inhibitory activity compared to kojic acid. A phenylalanine residue with a hydrophobic side chain at the first position near kojic acid exhibited better inhibitory activity than either lysine with a positively charged side chain or tyrosine with a polar side chain. This suggests that hydrophobic side chains might contribute to their binding in the hydrophobic pocket near the active site of TYR. When the phenylalanine at the first position was replaced with tryptophan, inhibitory activity was decreased. Interestingly, placing a tyrosine residue at the third position induced an approximately 10-fold increase in the inhibitory activity. In addition, storage stabilities of amino acid derivatives of kojic acid were approximately 15 times higher and their toxicity was lower than that of kojic acid.

Summary

As with peptidic inhibitors of lipid peroxidation, length, amino acid sequence and conformation determine the strength and mechanism of TYR inhibition. Most of the potent antibrowning peptides described in literature contain the amino acid residue Cys. In general Cys, and other thiol-containing compounds, are claimed to be potent inhibitors of melanin formation. Controversially, Cys plays an important role in pigmentation and the formation of pheomelanin (Figure 5.3). Hence, Cys and other thiols only change the course of melanogenesis toward stable adducts or lighter pigments, away from dark pigments, and the activity of TYR remains unaffected. Although this adduct formation is the main mechanism of inhibition by thiol-containing compounds, it is also proposed that they are able to chelate copper in the active site of TYR. Also His is known as a copper-chelating residue. This interaction is likely to result in a more specific inhibition of TYR, depending on the other amino acids present in the peptide or protein. Apart from thiol-containing amino acids, other amino acids free in solution have not shown a significant inhibitory effect on the TYR reaction (Table 5.2). However, when incorporated within a peptide or protein sequence, the presence of these residues may become important. Considering the hydrophobic active site of PPOs,
hydrophobic residues may establish an interaction between a peptide and the active site of the enzyme. Indeed the cyclic peptides reported to inhibit TYR are very hydrophobic in nature containing mainly Pro, Gly, Leu, and Phe. In all known type 3 copper proteins, the entrance of the active site is covered by an amino acid, which has to be removed for activation (Decker, 2007). This blocking residue is reported to be either a hydrophobic Phe or Leu, or a Tyr depending on the type of copper protein. The side chains of the blocking residues are positioned at the active site, but in case of Tyr kept at a certain distance to avoid reaction. Peptide mimics of the protein domains containing these blocking residues, may be potential inhibitors of TYR.
Chapter 6

Novel peptides with tyrosinase inhibitory activity

Abstract – Tyrosinase (TYR) inhibition by peptides may find its application in food, cosmetics or medicine. In order to identify novel TYR inhibitory peptides, protein-based peptide libraries made by SPOT synthesis were used to screen for peptides that show direct interaction with TYR. One of the peptide libraries studied consists of overlapping, octameric peptides derived from industrial proteins as β-casein, α-lactalbumin, β-lactoglobulin, ovalbumin, gliadin, glycine, and β-conglycinin. On-membrane activity staining resulted in a set of peptides that are not only able to bind to TYR, but are able to inhibit TYR as well. Peptides containing aspartic or glutamic acid residues usually do not bind very well to TYR. Strong TYR-binding peptides always contain one or more arginine residues, often in combination with phenylalanine, while lysine residues can be found equally among non-binding peptides as well as moderate TYR-binding peptides. The presence of the hydrophobic, aliphatic residues valine, alanine or leucine appears to be important for TYR inhibition. Therefore, good TYR inhibitory peptides preferably contain arginine and/or phenylalanine in combination with valine, alanine and/or leucine.
Tyrosinase inhibitory peptides

Introduction

Tyrosinase (TYR) is a copper-containing enzyme that catalyzes the ortho-hydroxylation of monophenols to catechols and their subsequent oxidation to ortho-quinones. The ubiquitous enzyme initiates the synthesis of melanin and is responsible for the browning that occurs upon bruising or long-term storage of vegetables, fruits, and mushrooms. In mammals, TYR is not only responsible for browning of hair and skin pigmentation (del Marmol, 1996; Sturm, 2001), but also for skin anomalies such as hypo (vitiligo) or hyper (flecks or freckles) pigmentation (Piamphongsant, 1998). Furthermore, TYR may play a role in cancer and neurodegenerative diseases, such as Parkinson’s disease (Cavaliere, 2002). As TYR is quite a significant target in the fields of agriculture, food, medicine, and cosmetics, the development and screening of potent inhibitors of TYR has received much attention (Iyengar, 1992; Kim, 2005a; Seo, 2003). There are many potent TYR inhibitors known today, for example sulfite or kojic acid (Chen, 1991a), but their use is being restricted. Adverse side effects, e.g. high toxicity toward cells and low stability toward oxygen and water, limit their application. Safety is a primary consideration for inhibitors, especially for those in food and cosmetic products. TYR inhibitors from natural sources have great potential, as they are considered to be safe and largely free from adverse side effects. Therefore, TYR inhibition by proteins and protein hydrolysates as well as individual peptides and amino acids has been investigated. Proteins and peptides from natural resources such as milk (Chen, 2006; Nakajima, 1996; Tomita, 1993), wheat (Okot-Kotber, 2001), honey (Ates, 2001; Oszmianski, 1990), silk (Kato, 1998), and the housefly (Daquinag, 1995; Daquinag, 1999) appeared to be able to inhibit TYR activity. Other TYR inhibitory peptides investigated are cyclic peptides (Morita, 1994), kojic acid-tripeptides (Kim, 2004), and dipeptides (Girelli, 2004). From the individual amino acids, Cys appeared to be the best TYR inhibitor (Kahn, 1985a). However, the inhibition observed is due to conjugation of Cys with the enzymatically produced quinone (Dudley, 1989), rather than an enzyme inhibition. The natural tripeptide glutathione inhibits melanin synthesis in a similar way by formation of glutathionylDOPAquinone adducts (Villarama, 2005). This type of inhibition does not affect the activity of TYR and is therefore not suitable for most applications.

In this study, the SPOT synthesis approach was used to identify peptides that are able to inhibit TYR from Agaricus bisporus through a direct interaction with the enzyme. By using SPOT synthesis (Frank, 1992; Frank, 2002; Schurink, 2006) a large peptide library composed of octameric peptides from different industrial protein sources, such as milk
(β-casein, α-lactalbumin, and β-lactoglobulin), egg (ovalbumin), wheat (gliadin), and soy (glycinin, and β-conglycinin), was synthesized. Besides this, fragments from the phenol oxidase inhibitor (POI) protein, from the hemolymph of the housefly (Daquinag, 1995; Daquinag, 1999), were synthesized to determine which region of the POI protein may be responsible for inhibition of TYR. The peptide libraries were screened consecutively for TYR-peptide interaction and TYR inhibition.

Experimental procedures

Purification of mushroom TYR
A crude preparation of TYR from *Agaricus bisporus* (EC 1.14.18.1, Sigma-Aldrich, Zwijndrecht, The Netherlands) was purified by size exclusion chromatography. The gel filtration column (HiPrep 16/60, Sephacryl S-300, High Resolution, Code No. 17-1167-01, Lot. No. 245814, Pharmacia Biotech) was calibrated with protein standards of known molecular mass. A 1 mL solution of crude TYR (10 mg mL⁻¹) in 50 mM sodium phosphate pH 6.5 was loaded onto the column and eluted with a flow rate of 1 mL min⁻¹. Fractions of 2 mL were collected and stored at −20°C.

Activity measurement of TYR
TYR activity was determined spectrophotometrically by measuring the oxygen-dependent conversion of L-3,4-dihydroxyphenylalanine (L-DOPA, Sigma-Aldrich, Zwijndrecht, The Netherlands) in the presence of an excess 3-methyl-2-benzothiazolinone hydrazone (MBTH, Fluka Chemie, Zwijndrecht, The Netherlands). L-DOPA is oxidized by TYR to form a quinone, which immediately reacts with MBTH to form a red colored complex (Espín, 1995; Espín, 1997). In a 1 mL cuvet 100 µL of 50 mM MBTH was added to 800 µL 18 mM L-DOPA in 100 mM sodium phosphate pH 6.5 saturated with air. The reaction was started with 100 µL 5 µg mL⁻¹ TYR and the increase in absorbance was measured at 484 nm and 30°C. The initial rate \( v \) in µmol L-DOPA per min was calculated from the steepest linear part of the curve using the following formula: \( v (\mu mol \min^{-1}) = 1000 \ (\mu L) \times 2 \times \text{slope } (\Delta A \min^{-1}) / (\varepsilon \ (M^{-1} \ cm^{-1}) \times 1 \ (cm)) \), where \( \varepsilon = 22300 \ M^{-1} \ cm^{-1} \) is the molar extinction coefficient of the red colored complex at 484 nm (Espín, 1997). Commercial TYR from *Agaricus bisporus* has a specific activity of 44 ± 4 µmol min⁻¹ mg⁻¹ and a \( K_M \) for L-DOPA of 0.66 ± 0.06 mM (Figure 6.1).
Tyrosinase inhibitory peptides

Figure 6.1 – Michaelis-Menten kinetics of L-DOPA oxidation by TYR at pH 6.5 and 30°C. $K_M = 0.66 \pm 0.06$ mM L-DOPA, which is in agreement with literature (Espín, 2000b; Fenoll, 2002), and $V_{max} = 22.3 \pm 0.36$ nmol min$^{-1}$ at 0.5 µg crude TYR in the assay.

**In-gel digestion of TYR and extraction for peptide mass fingerprinting**

Coomassie stained bands of the two protein fragments, of the purified TYR preparation, were cut out of the SDS-PAGE gel for peptide mass fingerprinting. The gels were washed with milliQ water and subsequently with acetonitrile : 50 mM NH$_4$HCO$_3$ pH 8.0 (1 : 1) to remove the Coomassie dye. The gel pieces were frozen and defrozen in order to increase the protein access area and subsequently dried in a vacuum centrifuge. The cysteines were reduced during at least 1 hour at 56°C, after adding 50 mM dithiothreitol in 50 mM NH$_4$HCO$_3$ pH 8.0. After cooling to room temperature and washing once with 50 mM NH$_4$HCO$_3$ pH 8.0, the cysteines were alkylated, by adding 10 µL 55 mM iodoacetamide in 50 mM NH$_4$HCO$_3$ pH 8.0, during at least 45 min in the dark with occasional vortexing. Then the gel pieces were washed thoroughly with 50 mM NH$_4$HCO$_3$ pH 8.0 and dried in the vacuum centrifuge in 30 to 120 min. The gels were rehydrated with 5 µL cold trypsin (bovine sequencing grade, Roche, Mannheim, Germany) 50 ng µL$^{-1}$ in 50 mM NH$_4$HCO$_3$ pH 8.0 and incubated overnight while shaking at room temperature. The liquid phase containing the peptides was collected and the remaining gel pieces were extracted with 10 µL 50 mM NH$_4$HCO$_3$ pH 8.0 with 5% trifluoroacetic acid (TFA) followed by 10 µL 10% acetonitrile with 1% TFA and combined with the liquid phase. The samples were analyzed by LC-MS. An electrospray potential of 1.8 kV
was applied with a capillary temperature of 150°C. Full scan positive mode MS spectra with 3 microscans were measured between \( m/z \) 350 and 1400 on a LCQ classic (Thermo electron, San Jose, USA). MS/MS scans of the three most abundant peaks in the MS scan were recorded in data dependent mode at 35% collision energy with a 4 D isolation width. Each run with all MS/MS spectra obtained was analyzed with Bioworks 3.2 using a database containing all relevant protein sequences.

**SPOT synthesis of the peptide libraries**

Peptides derived from the sequences of POI (Daquinag, 1995; Daquinag, 1999), \( \beta \)-casein (SwissProt accession no. P02666), \( \alpha \)-lactalbumin (SwissProt accession no. P00711), \( \beta \)-lactoglobulin (SwissProt accession no. P02754), ovalbumin (RefSeq accession no. NP_990483), gliadin (SwissProt accession no. P02863), glycine (SwissProt accession no. P13916), and \( \beta \)-conglycinin (SwissProt accession no. P13916) were synthesized in duplicate on derivatized cellulose membranes (Amino-PEG membrane, substitution 400 nmol cm\(^{-2}\), Intavis AG, Bergisch Gladbach, Germany) using the standard 9-fluorenylmethoxycarbonyl (Fmoc) strategy. The activated amino acid derivatives were delivered on defined positions on the membranes using a pipetting robot (Autospot upgrade kit for the Automated Multiple Peptide Synthesizer AMS 422, software AutoSpot A, Abimed Analysen-Technik, Langenfeld, Germany). When the desired peptides were fully assembled the membranes were dried and stored under vacuum at –20°C until use.

**Fluorescent labeling of TYR**

Purified TYR fractions with the highest TYR activity containing ±0.25 mg mL\(^{-1}\) TYR were labeled with Marina Blue (MB, FluorReporter Protein Labeling Kit F-10230, Molecular Probes, Leiden, The Netherlands). In total 3 mg of pure TYR was labeled with 0.5 mg MB. After separation of the reactants using PD-10 desalting columns (Amersham Biosciences, Uppsala, Sweden) the average degree of labeling of the TYR-MB preparation was ~5 labels per protein molecule, as determined by MB absorption at 365 nm. The labeled protein was analyzed by SDS-PAGE to confirm that the label is covalently linked to the protein. The enzyme activity was not affected by the labeling procedure.

**Incubation of the cellulose-bound peptides with TYR-MB**

The peptide libraries were pre-incubated in 100 mL 50 mM sodium phosphate pH 6.5. The background fluorescence of the membranes was recorded with excitation at 365 nm using the FluorChem 8800 (Alpha Innotech Corporation, Hoechst Blue HB-500 emission filter 465
nm, AlphaEaseFC™ Software Version 3.1.2), before adding TYR-MB (5 mL 0.15 mg mL⁻¹) to each membrane. The membranes were incubated in the dark for about 1 hour with TYR-MB, while gently shaken at room temperature. Then the membranes were washed 3 times with buffer and the fluorescence of the bound TYR-MB was recorded using the same settings as for the background. The light density of each spot on the membranes was measured on a linear scale from 0-100%, with 0% = black (no light) and 100% = white (maximum light yield) at an illumination time of 16 s. The background fluorescence was subtracted to obtain the fluorescence intensity resulting from the bound TYR-MB only.

**On-membrane activity staining of bound TYR-MB**

Immediately after recording the fluorescence of the bound TYR-MB, the membranes were homogeneously sprayed with a freshly prepared mixture of 50 mM MBTH : 18 mM L-DOPA (1 : 8). When the enzymatically initiated red color was developed, the membranes were gently washed with milliQ water to remove excess of substrate and to stop the reaction. After drying under vacuum, the membranes were scanned for analysis and sealed in plastic for conservation. The relative intensities of the red color on the spots were measured using the same software as for the intensities of TYR-MB fluorescence.

![Figure 6.2](image_url)

**Figure 6.2** – Elution profile of the gel filtration of TYR. Fractions of 2 mL were collected and analyzed for protein concentration $c$ (●) and TYR activity $v$ (○). TYR elutes at 65 mL. The specific activity increased from $44 \pm 4 \mu$mol min⁻¹ mg⁻¹ before to $331 \pm 11 \mu$mol min⁻¹ mg⁻¹ after purification. According to calibration series, TYR has a molecular mass of $127 \pm 5$ kD.
Results

**TYR purification and labeling**

The commercial mushroom TYR preparation was purified in order to screen the peptide libraries for peptides that bind to TYR. Peptides that interact with TYR are potential inhibitors of TYR. In this approach it is of great importance to remove any other protein present in the TYR preparation to avoid false-positive results. A purification of 7.5 times was obtained by a single gel filtration step (Figure 6.2) resulting in a specific activity of $331 \pm 11$ µmol min$^{-1}$ mg$^{-1}$.

Based on its elution behavior compared to standard proteins, the apparent molecular mass of native TYR was estimated to be around $127 \pm 5$ kD.

![Figure 6.3](image)

**Figure 6.3** – (A) SDS-PAGE of TYR. From left to right: marker proteins (lane 1, molecular weight indicated in kD), crude TYR (lane 2), TYR-MB (lane 3), and TYR (lane 4). The right part of the figure shows the fluorescence of TYR-MB at 365 nm before staining. The two bands, H and L, are $49 \pm 1.7$ kD and $14 \pm 0.5$ kD, respectively. (B) Absorption spectrum of TYR-MB (--) and TYR (--) at 0.25 mg mL$^{-1}$. TYR-MB has an absorbance maximum at 365 nm. The molar extinction coefficient of purified TYR at 280 nm is estimated be around $27 \cdot 10^4$ M$^{-1}$ cm$^{-1}$.

Fractions with the highest TYR activity were used for labeling with Marina Blue (MB). Fluorescent labeling of TYR allowed on-membrane detection of the TYR-peptide interaction. Analysis of the fluorescent TYR-MB by SDS-PAGE clearly revealed the presence of a high (H) and low (L) molecular mass chain (Figure 6.3). The H chain has an apparent molecular mass of $49 \pm 1.7$ kD, whereas the L chain has an apparent molecular mass of $14 \pm 0.5$ kD. Assuming a heterotetrameric quaternary structure of (HL)$_2$, the molecular mass of native TYR would be around $126 \pm 4$ kD. This is in agreement with the gel filtration results and with the structure described for *Agaricus bisporus* TYR (van Gelder, 1997; Strothkamp, 1976).
Tyrosinase inhibitory peptides

In order to determine the origin of both H and L chains, the protein was blotted onto a PVDF membrane for N-terminal sequence analysis by Edman degradation (Midwest Analytical Inc., St. Louis, USA). The first 12 amino acid residues of the L chain were identified to be ATNSGTLIIFDQ. This fragment does not match the known protein sequences of TYR from *Agaricus bisporus* strain U1 (PPO1 and PPO2, SwissProt accession nos. Q00024 and O42713 respectively) (Wichers, 2003). The N-terminal sequence of the H chain could not be determined since it appeared to be blocked. Peptide mass fingerprinting of the H chain showed unambiguously the presence of two fragments, FFTLYVR (Xcorr = 2.3) and LNIVDFVKNEKFFTLYVR (Xcorr = 3.6), that are both identical to the N-terminal part of PPO2 isoenzyme. MS/MS scans of other trypsine fragments of the H chain did not correspond to PPO1 or PPO2. Also, peptide fragments of the L chain showed no coverage with any of the known TYR sequences.

**TYR binding and inhibition by peptides derived from POI**

The POI derived peptide library consists of overlapping peptides with a frame shift of one amino acid (fine scan), ranging from ten down to six residues in length, covering the complete sequence of POI (Daquinag, 1995; Daquinag, 1999). The POI derived peptide library was incubated with TYR-MB to screen for TYR-peptide interactions. Accumulation of TYR-MB at certain spots on the membrane was visualized by illumination with UV light (Figure 6.4A). After correction for the background fluorescence, the resulting, relative fluorescence intensity is directly related to the amount of bound TYR-MB at certain spots on the membrane. All the peptides that show a relative fluorescence of bound TYR-MB above 80% contain the motive TKR. From the fine scan an average contribution of each individual amino acid residue in the TYR-peptide interaction was calculated (Figure 6.5A). Especially from the shorter peptides, it is evident that the presence of an Arg residue is very important for the TYR-peptide interaction. Since nearly all peptides in the POI library contain a Lys residue, the role of Lys in TYR-peptide interaction cannot be established. For longer peptides the effect of Arg is less pronounced, since it is nearly averaged out by the presence of amino acid residues that are less favorable for the TYR-peptide interaction. The presence of a negatively charged residue Asp and possibly also Glu is highly unfavorable for the TYR-peptide interaction.
Figure 6.4 – POI-derived peptide library. The library contains a duplicate set of overlapping peptides of different length, covering the complete sequence of POI, that are covalently bound to a cellulose membrane. (A) Negative image of the peptide library at 365 nm before binding of TYR-MB (left, faint background fluorescence only) and after binding of TYR-MB (right, TYR-MB fluorescence on top of background fluorescence). TYR-MB is binding to certain peptides on the membrane. After correction for the background, signal intensity represents the amount of TYR-MB bound at each spot. (B) Activity staining of TYR-MB bound to POI derived peptides. The color of the spots is the actual color of the complex formed by reaction between the enzymatically formed quinone product and MBTH (see cover).

On-membrane activity staining of bound TYR-MB (Figure 6.4B) was performed to observe direct inhibition by the POI derived peptides on the membrane. The activity observed depends on the amount of enzyme bound and the inhibiting effect of the peptides. The ratio between the activity \((A)\) and the fluorescence yield \((F)\) gives a measure for the specific activity of bound TYR-MB. A plot of the specific activity \((A/F)\) against the fluorescence intensity \((F)\) showed a clear correlation between the specific activity observed and the amount of TYR-MB bound to the spots (plot not shown). The majority of the peptides are supposed not to affect the TYR activity significantly and the following empirical relation can be derived for the POI peptide library: 

\[
(A/F)_{\text{exp}} = 23.7 \times (F)^{-0.71},
\]

wherein \((A/F)_{\text{exp}}\) is the specific activity expected for a certain amount of TYR-MB bound to a spot containing a few
nmol of a peptide that has neither an inhibiting nor an activating effect on TYR. All Tyr-containing peptides and peptides from which the specific activity observed deviate significantly from the majority of the peptides, at a certain strength of TYR-binding, were not considered when this equation was derived. Tyr-containing peptides are substrates for TYR and thus may contribute to an apparent activation of TYR (Girelli, 2004).

Figure 6.5 – Average contribution of individual amino acids within peptides derived from POI (Daquinag, 1995; Daquinag, 1999) in binding to TYR and inhibition of TYR. (A) Relative fluorescence signal ($F$) of TYR-MB binding to POI-derived peptides ranging from ten down to six residues in length. Maximum TYR binding was observed for peptides of six amino acid residues in length containing Arg. (B) Relative inhibition ($I$) of TYR by POI-derived peptides that show interaction with TYR. Maximum TYR inhibition was observed for peptides of six amino acid residues in length containing Val. Note that the relatively strong inhibition of the peptides at the N-terminus of POI is less relevant, since there is only weak binding of TYR by these peptides, and is therefore left out.

TYR-binding peptides that decrease the specific activity of TYR significantly compared to the specific activity expected are considered to be inhibitors of TYR. The strength of inhibition ($I$) is the difference between the specific activity expected ($A F^{-1}$)exp and the specific activity
observed \((A F^{-1})_{\text{obs}}\) expressed as a percentage of the specific activity expected: \(I = 100 - \left(\frac{(A F^{-1})_{\text{obs}}}{(A F^{-1})_{\text{exp}}} \times 100\right)\). In this way a relative measure for the TYR inhibition could be calculated independently of the amount of bound TYR. All the peptides that show a relative inhibition of TYR above 80% (and a relative fluorescence of bound TYR-MB of at least 10%) contain either the motive EIVP or DVCC. Similar to the analysis of the binding data from the POI library, an average contribution of each individual amino acid residue to TYR inhibition by a certain peptide was calculated (Figure 6.5B). From this it appears that especially the amino acid residue Val is important for the observed inhibition of TYR by POI derived peptides.

**TYR binding and inhibition by peptides derived from industrial proteins**

Besides the main purpose to identify novel TYR inhibitors, a large peptide library containing a variety of different peptides derived from industrial protein sources was screened to get more insight in the importance of certain amino acids in the TYR-peptide interaction and the resulting TYR inhibition. The industrial protein derived peptide library consists of overlapping peptides with a frame shift of four amino acids and a length of eight amino acids, covering the complete sequence of \(\beta\)-casein, \(\alpha\)-lactalbumin, \(\beta\)-lactoglobulin, ovalbumin, gliadin, glycinin, and \(\beta\)-conglycinin. The industrial protein derived peptide library was treated similarly to the POI peptide library (pictures not shown). For the industrial protein peptide library the empirical relation between the specific activity expected and the amount of bound TYR was derived to be: \((A F^{-1})_{\text{exp}} = 34.7 \times (F)^{-0.79}\). The relative inhibition was calculated in the same way as for the POI peptide library.

An overview of TYR binding and inhibition (expressed as \(F\) and \(I\), respectively) determined for all peptides in the industrial protein library is presented in distribution plots (Figure 6.6). From these plots it can be seen that the best TYR-binding peptides (located on the right) contain an Arg residue and that most TYR-inhibiting peptides (that also bind to TYR, located on the top right corner) contain a Val residue. The exact location of Arg and/or Val-containing peptides depends on the presence of other, neighboring amino acid residues within these peptides. Peptides containing a negatively charged amino acid residue Asp or Glu are highly unfavorable for the TYR-peptide interaction and are, therefore, mainly located on the left of the distribution plot. Most peptides containing Tyr are located on the bottom of the distribution plot, showing an apparent activation of TYR, since they may be converted by TYR and hence contribute to an increase in TYR activity observed (Girelli, 2004). These results, regarding the importance of amino acid residues within TYR-inhibiting peptides
Tyrosinase inhibitory peptides obtained from the industrial protein peptide library, are in accordance with the results obtained from the POI peptide library.

**Figure 6.6** – Distribution of the octameric peptides derived from industrial proteins in plots of TYR inhibition ($I$) versus TYR binding ($F$). (A) Location of peptides containing Asp and/or Glu, Arg, Tyr and Val indicated by dark circles. The open circles represent all other peptides present in the library. Strong TYR-binding peptides are located on the right and strong TYR-inhibiting peptides are located on the top. Nonbinding peptides or peptides with a relative error larger than 100% of the inhibition are not shown in these plots. (B) Peptides that are binding to and inhibiting TYR. The best TYR-binding and inhibiting peptides ($F > 20\%$ and $I > 10\%$) are indicated with their spot number (Table 6.1).
Table 6.1 – Peptides derived from industrial proteins that are binding (F) to TYR and simultaneously inhibit (I) the TYR activity.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Sequence</th>
<th>Protein source</th>
<th>F / %</th>
<th>I / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>145</td>
<td>QINKVVRF</td>
<td>Ovalbumin</td>
<td>100 ± 29.2</td>
<td>20.1 ± 7.45</td>
</tr>
<tr>
<td>347</td>
<td>PFPRPQPQR</td>
<td>β-L-Conglycinin</td>
<td>99.4 ± 10.7</td>
<td>4.95 ± 1.19</td>
</tr>
<tr>
<td>339</td>
<td>IPRRPRPR</td>
<td>β-L-Conglycinin</td>
<td>96.8 ± 3.51</td>
<td>1.85 ± 0.37</td>
</tr>
<tr>
<td>171</td>
<td>IIRNVLOP</td>
<td>Ovalbumin</td>
<td>96.2 ± 8.11</td>
<td>15.9 ± 2.45</td>
</tr>
<tr>
<td>186</td>
<td>LFRVASMA</td>
<td>Ovalbumin</td>
<td>84.9 ± 2.81</td>
<td>34.9 ± 0.47</td>
</tr>
<tr>
<td>251</td>
<td>PLGQGSFR</td>
<td>Gliadin</td>
<td>82.6 ± 3.80</td>
<td>3.87 ± 0.22</td>
</tr>
<tr>
<td>200</td>
<td>VRFPVQL</td>
<td>Gliadin</td>
<td>79.2 ± 3.07</td>
<td>8.06 ± 0.42</td>
</tr>
<tr>
<td>276</td>
<td>TLRNALR</td>
<td>Glycinin</td>
<td>76.9 ± 17.3</td>
<td>18.3 ± 4.56</td>
</tr>
<tr>
<td>51</td>
<td>VRGPFPPII</td>
<td>β-L-Casein</td>
<td>75.5 ± 5.94</td>
<td>9.86 ± 1.98</td>
</tr>
<tr>
<td>146</td>
<td>VVRFDKL</td>
<td>Ovalbumin</td>
<td>75.2 ± 14.5</td>
<td>11.3 ± 5.33</td>
</tr>
</tbody>
</table>

Strong TYR-binding peptides (F > 75% and I > 0%)

<table>
<thead>
<tr>
<th>Spot</th>
<th>Sequence</th>
<th>Protein source</th>
<th>F / %</th>
<th>I / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>APLRVYVE</td>
<td>β-Lactoglobulin</td>
<td>13.3 ± 0.12</td>
<td>96.8 ± 36.9</td>
</tr>
<tr>
<td>54</td>
<td>VSLLLVGI</td>
<td>α-Lactalbumin</td>
<td>21.2 ± 1.54</td>
<td>90.8 ± 2.46</td>
</tr>
<tr>
<td>325</td>
<td>ASVSVSFG</td>
<td>β-L-Conglycinin</td>
<td>12.0 ± 6.61</td>
<td>90.2 ± 7.77</td>
</tr>
<tr>
<td>195</td>
<td>MKTLILFLV</td>
<td>Gliadin</td>
<td>14.2 ± 0.84</td>
<td>89.7 ± 12.0</td>
</tr>
<tr>
<td>151</td>
<td>SVINHSVSL</td>
<td>Ovalbumin</td>
<td>15.8 ± 0.20</td>
<td>88.3 ± 2.65</td>
</tr>
<tr>
<td>175</td>
<td>MVLVNAIV</td>
<td>Ovalbumin</td>
<td>16.4 ± 1.04</td>
<td>87.7 ± 0.07</td>
</tr>
<tr>
<td>53</td>
<td>MMSFVSLLL</td>
<td>α-Lactalbumin</td>
<td>21.7 ± 3.88</td>
<td>87.3 ± 0.44</td>
</tr>
<tr>
<td>140</td>
<td>IAIMSALA</td>
<td>Ovalbumin</td>
<td>18.2 ± 0.97</td>
<td>86.6 ± 0.19</td>
</tr>
<tr>
<td>99</td>
<td>ISLLDAQS</td>
<td>β-Lactoglobulin</td>
<td>10.5 ± 0.29</td>
<td>83.4 ± 0.02</td>
</tr>
<tr>
<td>196</td>
<td>VVRFDKL</td>
<td>Ovalbumin</td>
<td>25.7 ± 0.38</td>
<td>82.9 ± 0.05</td>
</tr>
</tbody>
</table>

Strong TYR-inhibiting peptides (F > 10% and I > 80%)

<table>
<thead>
<tr>
<th>Spot</th>
<th>Sequence</th>
<th>Protein source</th>
<th>F / %</th>
<th>I / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>186</td>
<td>LFRVASMA</td>
<td>Ovalbumin</td>
<td>84.9 ± 2.81</td>
<td>34.9 ± 0.47</td>
</tr>
<tr>
<td>196</td>
<td>LILVLLAI</td>
<td>Gliadin</td>
<td>25.7 ± 0.38</td>
<td>82.9 ± 0.05</td>
</tr>
<tr>
<td>54</td>
<td>VSLLLVGI</td>
<td>α-Lactalbumin</td>
<td>21.2 ± 1.54</td>
<td>90.8 ± 2.46</td>
</tr>
<tr>
<td>53</td>
<td>MMSFVSLLL</td>
<td>α-Lactalbumin</td>
<td>21.7 ± 3.88</td>
<td>87.3 ± 0.44</td>
</tr>
<tr>
<td>145</td>
<td>QINKVVRF</td>
<td>Ovalbumin</td>
<td>100 ± 29.2</td>
<td>20.1 ± 7.45</td>
</tr>
<tr>
<td>323</td>
<td>LLLGLVFL</td>
<td>β-L-Conglycinin</td>
<td>23.3 ± 1.83</td>
<td>68.1 ± 0.94</td>
</tr>
<tr>
<td>171</td>
<td>IIRNVLOP</td>
<td>Ovalbumin</td>
<td>96.2 ± 8.11</td>
<td>15.9 ± 2.45</td>
</tr>
<tr>
<td>55</td>
<td>LVGILFHA</td>
<td>α-Lactalbumin</td>
<td>25.1 ± 1.36</td>
<td>61.8 ± 0.60</td>
</tr>
<tr>
<td>276</td>
<td>TLNRLNAR</td>
<td>Glycinin</td>
<td>76.9 ± 17.3</td>
<td>18.3 ± 4.56</td>
</tr>
<tr>
<td>121</td>
<td>LACQLCLVR</td>
<td>β-Lactoglobulin</td>
<td>32.7 ± 8.78</td>
<td>39.0 ± 14.2</td>
</tr>
<tr>
<td>146</td>
<td>VVRFDKL</td>
<td>Ovalbumin</td>
<td>75.2 ± 14.5</td>
<td>11.3 ± 5.33</td>
</tr>
<tr>
<td>252</td>
<td>GSFRPSEQ</td>
<td>Gliadin</td>
<td>56.8 ± 7.97</td>
<td>10.6 ± 1.78</td>
</tr>
<tr>
<td>176</td>
<td>NAIVFKGL</td>
<td>Ovalbumin</td>
<td>29.3 ± 2.43</td>
<td>28.8 ± 2.63</td>
</tr>
<tr>
<td>170</td>
<td>QTNGRNN</td>
<td>Ovalbumin</td>
<td>40.7 ± 6.46</td>
<td>14.4 ± 4.55</td>
</tr>
<tr>
<td>181</td>
<td>MPFRVTIEQ</td>
<td>Ovalbumin</td>
<td>32.2 ± 6.61</td>
<td>15.7 ± 4.63</td>
</tr>
<tr>
<td>232</td>
<td>VVLQHNNI</td>
<td>Gliadin</td>
<td>28.6 ± 1.94</td>
<td>12.7 ± 0.52</td>
</tr>
</tbody>
</table>

The best TYR-binding and TYR-inhibiting peptides are located on the top right corner of the distribution plot (Figure 6.6B) and originate from different protein sources, but most of them are derived from ovalbumin (Table 6.1). Strong TYR-binding peptides (F > 75% and I > 0%) always contain at least one Arg residue, often in combination with a Phe residue. Strong TYR-inhibiting peptides (F > 10% and I > 80%) contain (combinations of) Val, Ala and/or Leu residues and are therefore hydrophobic in nature. Good TYR-binding and inhibiting peptides usually have a combination of Arg and/or Phe with Val, Ala and/or Leu, for example peptide 186 with the sequence LFRVASMA from ovalbumin. The three best TYR-inhibiting peptides

Chapter 6
from the industrial protein peptide library with $F > 20\%$ are the overlapping peptides 53 and 54, MMSFVSSL and VSLLVGI respectively, from $\alpha$-lactalbumin and peptide 196, LILVLLAI, from gliadin.

**Discussion**

**TYR structure**

Many primary structures of fungal TYRs have been reported (Halaouli, 2006) and much is known about the TYR catalytic center (Matoba, 2006). For *Agaricus bisporus* TYR the quaternary structure remains elusive. During maturation and activation, proteolytic processing from the C-terminal side of the latent 67 kD TYR sequence eventually generates a 43 kD active TYR and a 15 kD fragment (Espín, 2000a), which may well correspond to the 49 ± 1.7 kD H chain and the 14 ± 0.5 kD L chain observed in the present study. In case of the composition (HL)$_2$ (van Gelder, 1997; Strothkamp, 1976), the molecular mass of the native enzyme would be 126 ± 4 kD (Figure 6.3A). This is in perfect agreement with the molecular mass of 127 ± 5 kD determined by gelfiltration (Figure 6.2).

The commercial TYR enzyme used in this study either originates from a third, unknown gene of strain U1 or from another *Agaricus bisporus* strain, since peptide mass fingerprinting of the H chain showed a low coverage of only 18 amino acid residues with PPO2 from strain U1. Despite this low coverage, it gives an indication that the commercial TYR resembles PPO2 probably more than PPO1. The L chain is proposed to be the product of C-terminal processing of TYR *in vivo* (Espín, 2000a). During this process a fragment, which size corresponds to the size of the L chain, is split off from the C-terminal part of TYR. The homology between the C-terminal parts of PPO1 and PPO2 is very low (Marusek, 2006). This may explain why the 12 amino acid residues identified by Edman degradation at the N-terminal end of the L chain show no homology with PPO1 or PPO2. Since the isolation procedure of the commercial TYR preparation is unknown, the possibility that the L chain originates from a different gene cannot be excluded. Information about the *Agaricus bisporus* genome is required to confirm whether or not the H and the L chain originate from another gene encoding a third TYR isoenzyme.
TYR binding and inhibition by peptides

Two independently analyzed peptide libraries were screened for peptides binding to and inhibiting TYR. From the POI peptide library it appeared that the only Arg residue (Figure 6.5A) present in POI plays an important role in the TYR-peptide interaction. Furthermore, good TYR-binding peptides containing Val (Figure 6.5B) are relatively good inhibitors of TYR. Although the Cys residues within the native POI protein are all involved in disulfide bridges (Daquinag, 1995; Daquinag, 1999), the average contribution of Val in TYR inhibition by peptides derived from POI may be affected by the adjacent, reduced Cys residues. However the importance of Val and other residues, regardless the effect of Cys residues, is confirmed with the results obtained from a larger peptide library containing a variety of peptides derived from different industrial protein sources. The results obtained with the POI derived peptide library are in agreement with the results obtained from the industrial protein derived peptide library.

Good TYR-binding peptides derived from industrial proteins usually contain the amino acid residues Arg and/or Phe (Figure 6.7A). Because Lys is not found to be important for the TYR-peptide interaction, it may be concluded that the interaction between TYR and the Arg-containing peptides is rather specific and not only based on electrostatics. The importance of Phe in the TYR-peptide interaction might be explained by the fact that Phe is structurally similar to Tyr, which is the natural substrate for TYR (see also below). However, the binding of Tyr-containing peptides cannot be established, since Tyr is oxidized upon incubation of the peptide library with TYR-MB. Apparently, peptides containing the DOPAquinone residue do not bind very well to TYR. Also peptides containing an Asp or Glu residue do not bind very well to TYR, suggesting that a negative charge is not favorable for TYR binding.

Good TYR-inhibiting peptides derived from industrial proteins usually contain a hydrophobic, aliphatic amino acid residue Val, Ala, Leu, Met and/or Ile (Figure 6.7B). The hydrophobic, aromatic residue Phe, which is relatively important in the TYR-peptide interaction, is less important in TYR inhibition. Apparently, despite its structural similarities with Tyr, Phe is presumably not binding to the active site of TYR. These findings are in accordance with results reported earlier, where Phe appears not to be a very good inhibitor of TYR (Farishian, 1980). Besides the hydrophobic residues mentioned above, peptides containing the polar, uncharged residues Cys and Ser, and to a minor extent Thr, are good TYR inhibitors as well. The TYR inhibition observed for peptides containing Ser and/or Thr is most probably similar to the mechanism of inhibition by Cys (Dudley, 1989; Villarama, 2005).
Tyrosinase inhibitory peptides

In some cases TYR-binding by peptides may result in an activation of TYR. This usually occurs with peptides containing the amino acid residues Tyr and/or Trp (Figure 6.7B). The activating effect of Trp containing peptides is less established, since there are only a few peptides in the library containing Trp. As explained in the results (sections 4.3.2 and 4.3.3), the presence of Tyr residues in TYR-binding peptides causes an apparent TYR activation since TYR oxidizes the Tyr residues. Of course, Tyr-containing peptides may still act like competitive inhibitors of L-DOPA oxidation by TYR, but the TYR activity and thus the
formation of quinones is not affected. Some relatively good TYR-binding peptides do not contain Tyr or Trp and are still activating TYR. An example of a relatively good TYR-binding peptide that simultaneously activates TYR is RINKKIEK. RINKKIEK is a lipoxygenase-inhibitory peptide identified from β-casein (Schurink, 2006). Although RINKKIEK is also binding to TYR, it appears to have an opposite effect on the activity of this enzyme.

Conclusions

Considering the relative fluorescence and the relative activity measured for TYR-MB binding to peptides within the libraries studied, conclusions can be drawn regarding the importance of certain amino acid residues within TYR-binding and TYR-inhibiting peptides. From both the POI and the industrial protein peptide library, it appeared that the presence of an Arg residue within a peptide is very beneficial for the TYR-peptide interaction. This is probably not only due to a simple electrostatic interaction, since the similarly charged amino acid residue Lys is not important for TYR binding at all. Obviously, the structure of the Arg side chain is required for a strong TYR-peptide interaction.

The observed TYR inhibition by peptides containing the polar, uncharged amino acid residues Cys or Ser is due to their ability to form adducts with the enzymatically formed DOPAquinone. Therefore peptides containing these residues as inhibitors of melanin formation are not very effective, since they are consumed in the reaction and the TYR activity is not affected. The presence of other amino acid residues, like the hydrophobic, aliphatic Val, Ala or Leu, is probably more important in inhibition of TYR by peptides, since they may inhibit the formation of quinones by direct interaction with TYR. Further experiments are needed to confirm and optimize the TYR-inhibiting activity of potential TYR-inhibiting peptides containing Arg and/or Phe in combination with Val, Ala and/or Leu.

Acknowledgements

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Chapter 7

General discussion
Summary

Uncontrolled oxidation processes initiated by enzymes such as lipoxygenase (LOX) and polyphenol oxidase (PPO) are the main cause of food quality decay. LOX is responsible for oxidation of polyunsaturated fatty acids (PUFAs) resulting in off-flavors and rancidity of fat-containing foods. PPO is an initiator of browning that occurs in, for example, fruits and vegetables. The use of additives able to inhibit or delay oxidative processes (antioxidants) is indispensable in preservation of food products. Besides deterioration of foods, LOXs are thought to play a role in various types of cancer, inflammatory diseases, cardiovascular diseases and neurodegenerative diseases (Chapter 2). PPOs are also involved in cancer, neurodegenerative processes, and are furthermore responsible for irregular hyperpigmentation disorders of the human skin (Chapter 5). Therefore, inhibition of these enzymes is not only an important target in food, but also in pharmaceutical and cosmetic industries. Many inhibitors of LOXs and PPOs are known to date. Although most of them are very potent inhibitors of the oxidation reactions catalyzed by these enzymes, they are unable to inactivate the enzyme directly. Most inhibitors known are polyphenols and they usually exert their function by scavenging of the intermediate radicals or oxidation products formed by the enzymes (chain-breaking antioxidants). The antioxidative inhibitors are consumed during the process leading to high concentrations required to significantly delay oxidation. Direct inhibition of the enzymes responsible (preventive antioxidants) would provide not only a more efficient, but also a more specific means to control undesired oxidation processes in food as well as the human body. Especially in the latter case, when applied as a therapeutic drug, this specificity is very important in order to minimize side effects. Besides lack of specificity, many known antioxidants are synthetic and/or toxic and are not allowed for use in food and cosmetic products. A preference for natural antioxidants has intensified the search for novel compounds able to inhibit the adverse oxidation reactions.

Over the course of years, proteins and peptides from various industrial sources have been shown to have antioxidant activity in several oxidation systems (Chapter 1), although the exact mechanism usually remained unclear. One major advantage of proteins and peptides derived from natural sources such as milk, egg, soy, and wheat is that they are generally regarded as safe and can readily be applied in foods and cosmetic products. Moreover, protein-protein interactions can be very specific and, therefore, proteins and peptides have the potential to inhibit LOX and PPO through a direct interaction with the enzyme. The objective of the research described in this thesis was to identify peptides as specific inhibitors...
of LOX from soybean (Glycine max) and tyrosinase (TYR), a PPO, from mushroom (Agaricus bisporus). A novel approach was chosen using synthetic peptides libraries, prepared via SPOT synthesis (Frank, 1992), in the identification of peptides having an interaction with these enzymes. Apart from a high-throughput format of screening, this method provides a tool to study the enzyme-peptide interaction in more detail via substitution analyses. Subsequent oxidation assays were performed in order to assess the inhibitory effect of the enzyme-binding peptides. Since the inhibition of two different oxidative enzymes was studied, this thesis is divided in two parts, the first concerning LOX inhibition and the second concerning TYR inhibition.

Chapter 3 describes the identification of peptides as LOX inhibitors from the milk protein β-casein. A peptide library composed of overlapping peptides based on the sequence of β-casein was prepared and screened for interaction with LOX-1 from soybean (Schurink, 2006). It appeared that peptides derived from five distinct regions of β-casein were able to bind to LOX-1 (Figure 3.2). Considering amino acid composition and sequence, there was no obvious correlation between these regions, except for the observation that a length of eight amino acid residues appears to be optimal for binding. Peptides previously isolated and identified from β-casein hydrolyzates were reported to inhibit the LOX-catalyzed oxidation of linoleic acid (LA) (Rival, 2001a; Rival, 2001b) and are located within two of these regions. The other three regions are new and were not detected via hydrolysis, demonstrating the advantage of screening of synthetic peptide libraries over protein hydrolyzates. Several binding and non-binding peptides were synthesized at larger scale via solid-phase peptide synthesis (SPPS) and their antioxidant activity was tested in solution-phase inhibition assays. In this study, different β-casein-derived peptides were identified to be able to inhibit the LOX-catalyzed oxidation of LA. Although the peptides are binding to LOX-1, an additional radical scavenging effect observed, for especially Tyr- or Trp-containing peptides, may explain the strong inhibitory effects of these peptides on the LOX-1 reaction. True LOX-1 inhibition resulting solely from an interaction with LOX-1, was observed for peptides derived from region 1 of β-casein, SITRINKK and especially RINKKIEK. LOX-1 inhibition kinetics with RINKKIEK revealed a noncompetitive inhibition with a $K_i$ between 150-200 $\mu$M, which is in the same range as values reported for common LOX inhibitors such as ascorbic acid, benzoic acid, and bisphenol A. In order to determine the importance of the amino acid residues within RINKKIEK for interaction with LOX-1 and the possibility to improve LOX-1 inhibition by such octapeptides, a substitutional analysis was carried out and the results are presented in Chapter 4. A new peptide library was composed of several RINKKIEK analogs and some
other β-casein-derived peptides. Considering all peptides present in the library, a clear relation between LOX-1 binding and the overall charge of a peptide was observed with +4 being most optimal for binding (Figure 4.4A). Several experiments demonstrated that the positively charged Lys residues appear to be crucial for the LOX-RINKKIEK interaction. Substitution of every residue within RINKKIEK, with one of the 19 other natural occurring amino acids, showed that nearly any replacement lowered the interaction with LOX-1 (Figure 4.7). This indicates that not only Lys residues, but also the other residues present in RINKKIEK are important for LOX-1 binding. However, scrambled peptides (for example IRKEIKKN) do not show a significantly lower affinity for LOX-1 and inhibit the enzyme to the same extent as RINKKIEK itself. Apparently the presence of these residues is more important than the position within the peptide sequence. Besides this, titration of LOX-1 with RINKKIEK as monitored by Trp-fluorescence quenching (Figure 4.5), indicates the presence of multiple binding sites for the peptide on the surface of LOX-1. Both observations imply that the LOX-RINKKIEK interaction is not very specific. Nevertheless, improvements to the RINKKIEK sequence were made by replacement of Glu. Most likely, replacement of the negatively charged residue by nearly any other residue, resulting in an overall charge of +4, improves binding as observed for the membrane-bound substitutional analogs of RINKKIEK (Figure 4.7). For both RINKKIPK and RINKKISK this increase in LOX-1 binding was accompanied by a nearly threefold increase in LOX-1 inhibition (Table 7.1). According to this study, the interaction between LOX-1 and the peptides is of low specificity and mainly based on electrostatics. The strength of inhibition is, however, higher than that of the common inhibitor L-carnosine. Although difficult to determine due to the complex oxidation system of LOX-1, the interaction between LOX-1 and the octameric peptides results in a noncompetitive inhibition of LOX-1 as described in Chapters 3 and 4.

Since there was only limited information available about inhibition of PPOs by industrial proteins or hydrolyzates thereof, binding and inhibition of TYR from mushroom was investigated using overlapping peptide arrays based on the sequence of proteins from several sources including the milk proteins β-casein, α-lactalbumin, and β-lactoglobulin, and furthermore egg ovalbumin, wheat gliadin, and two major soy proteins glycinin and β-conglycinin (Schurink, 2007). Besides this, a peptide library containing fragments of the phenoloxidase inhibitor (POI) from housefly was also analyzed. The libraries were screened for both TYR binding as well as TYR inhibition and the results are presented in Chapter 6. Strong TYR-binding peptides always contain an Arg residue, often in combination with Phe. In contrast to the results of LOX, there is no relation between peptide charge and enzyme
binding for TYR, although an overall negative charge disfavors binding. Strong TYR-inhibiting peptides contain the hydrophobic residues Val, Ala and/or Leu. Good TYR-binding and -inhibiting peptides, for example LFRVASMA from ovalbumin (Table 7.1), contains a combination of Arg and/or Phe with Val, Ala and/or Leu (Figure 6.7). It is possible that the hydrophobic residues of these peptides may block the active site of TYR in a similar way as described for the inhibiting domains, comprising hydrophobic blocking residues as Phe or Leu, of different type 3 copper proteins (Decker, 2007). Additional solution-phase assays are required to determine the mode of inhibition by these TYR-inhibiting peptides.

Table 7.1 – Selection of some important soybean LOX-1 and mushroom TYR-inhibiting peptides identified by using SPOT synthesis.

<table>
<thead>
<tr>
<th>Source</th>
<th>Protein Sequence</th>
<th>F (%)[^a]</th>
<th>I (%)[^b]</th>
<th>$K_d$ ($\mu$M)</th>
<th>$K_i$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide inhibitors of soybean LOX-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine milk</td>
<td>β-Casein</td>
<td>SITRINKK</td>
<td>66.3 ± 10.6</td>
<td>42.6 ± 0.92</td>
<td>137 ± 33.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RINKKIEK</td>
<td>85.9</td>
<td>57.7 ± 1.60</td>
<td>115 ± 40.7</td>
</tr>
<tr>
<td>Synthetic</td>
<td>-</td>
<td>RINKKIPK</td>
<td>85.0</td>
<td>59.8 ± 1.30</td>
<td>73.1 ± 8.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RINKKISK</td>
<td>66.9 ± 16.2</td>
<td>33.9 ± 1.09</td>
<td>60.8 ± 7.39</td>
</tr>
<tr>
<td>Peptide inhibitors of mushroom TYR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine milk</td>
<td>α-Lactalbumin</td>
<td>VSSLVGI</td>
<td>21.2 ± 1.54</td>
<td>90.8 ± 2.46</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMSFVSLL</td>
<td>21.7 ± 3.88</td>
<td>87.3 ± 0.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LVGILFHA</td>
<td>25.1 ± 1.36</td>
<td>61.8 ± 0.60</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>Ovalbumin</td>
<td>LFRVASMA</td>
<td>84.9 ± 2.81</td>
<td>34.9 ± 0.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>QINKVVRF</td>
<td>100 ± 29.2</td>
<td>20.1 ± 7.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IIIRNVLQP</td>
<td>96.2 ± 8.11</td>
<td>15.9 ± 2.45</td>
<td></td>
</tr>
<tr>
<td>Soy</td>
<td>Glycinin</td>
<td>TLNRNALR</td>
<td>76.9 ± 17.3</td>
<td>18.3 ± 4.56</td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>β-Conglycinin</td>
<td>LLLGLVFL</td>
<td>23.3 ± 1.83</td>
<td>68.1 ± 0.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gliadin</td>
<td>LILVLLAI</td>
<td>25.7 ± 0.38</td>
<td>82.9 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>

[^a] Relative binding intensities of the enzyme to the cellulose-bound peptides;[^b] Inhibition in the standard assay for LOX-1 and relative inhibition measured on the membrane for TYR.

Future perspectives

Taking into consideration the new results and insights obtained from the research described in this thesis it can be concluded that inhibition of the oxidative enzymes LOX and TYR is feasible by using peptides. Although the antioxidant capacity of these peptides lies within the same range as for some commonly known inhibitors, both strength and the intended specificity of inhibition is not yet sufficient for most application purposes. Since the inhibition of the peptides in this study was assessed using model enzymes in *in vitro* oxidation systems only, the results obtained are considered preliminary. Additional experiments need to be performed to determine the effect and stability of these peptides in more complex matrices such as food and/or whole cell systems. Other components present may interfere with the
enzyme-peptide interaction, especially when the specificity is low. With respect to the stability of peptidic inhibitors, it should be noted that the inhibitors are prone to degradation in the presence of proteases. On the other hand, considering production of antioxidant peptides, consecutive action of proteases and/or mixtures of proteases are required for preparative isolation of the active peptides from industrial proteins. For example, several RINKKIEK analogs from β-casein region 1 can be isolated by using different proteases (Figure 7.1).

Figure 7.1 – Possibilities for the isolation of LOX-inhibiting peptides from β-casein. Shown is the N-terminal part of β-casein including the RINKKIEK sequence (region 1) and the predicted cleavage sites of some useful proteases.

In view of several application purposes, the identification of potential, peptidic inhibitors of LOX and TYR was based on a direct interaction between the enzyme and peptide. Peptide libraries prepared by SPOT synthesis were used for high-throughput screening, analysis of molecular determinants and optimization of the enzyme-inhibitor interaction. So far this novel approach has never been used for the identification of antioxidant peptides and the current research has certainly proven its usefulness in this field. Although many peptides with antioxidant activity have been described in literature, it is for the first time that the observed antioxidant effect can be ascribed to a direct interaction between enzyme and peptide. Besides the high-throughput format of screening, SPOT synthesis and subsequent screening of the peptide libraries provides a powerful tool to study the molecular interaction on a fundamental level. In combination with quantitative structure-activity relationship (QSAR) (Collantes, 1995; Siebert, 2001), in silico docking (Hetényi, 2002), 3D-modeling studies, and/or co-crystallization experiments it may lead to new insights regarding the optimal length, amino acid composition, sequence and structure of an antioxidant peptide. Once identified and further characterized in appropriate (model) systems, new technological developments such as large-scale chromatography, membrane technologies, and selective extraction/precipitation procedures, open up possibilities for commercial production of antioxidant peptides from natural protein sources.
The work described in this thesis opens new perspectives for the identification and application of bioactive peptides. Bioactive peptides can be broadly applied in for example functional foods, nutraceuticals, cosmetics, and pharmaceuticals. Such peptides are currently isolated and purified from natural sources, manufactured using biotechnological or enzymatic production processes (Lombard, 2005), or chemically produced via liquid-phase or solid-phase synthesis (Andersson, 2000). Chemoenzymatic synthesis minimizes the use of protection groups and allows a large-scale production of bioactive peptides in a flexible, more economic way with shorter cycle times and increased throughput. An example of an enzymatically produced peptide is the artificial sweetener aspartame, which is a dipeptide applied as an ingredient of many consumer foods and beverages sold worldwide (Ager, 1998; Oyama, 1984). Since many processes in the human body are mediated by protein-protein interactions, such as receptor-ligand interactions in signal transduction pathways or antibody-antigen interactions within the immune system, the potential of peptides as therapeutics is intensifying. Market analysts estimate that the global market for peptide-based pharmaceuticals will expand at a growth rate nearly double the growth rate for pharmaceuticals overall (Marx, 2005). This development requires, however, dedicated methods for evaluation of the target activity along with manufacturing improvements, better stabilization and administration techniques. Human insulin, for example, is now manufactured for widespread clinical use using genetic engineering techniques (Shapley, 1982). Unlike many other, nonpeptidic medicines, insulin cannot be taken orally and is usually taken as subcutaneous injections. Like nearly all other proteins introduced into the gastrointestinal tract, insulin is reduced to fragments and single amino acid components, whereupon all bioactivity is lost. Research to develop encapsulation methods to ensure protection and controlled release delivery of such bioactive peptides may aid in counteracting such drawbacks (Hildebrand, 2000; Reithmeier, 2001). Integration of the method applied in this study for finding and optimizing bioactivity with technology for peptide manufacturing and administration will provide a new platform for bioactive peptides with application in food and health.
General discussion
Nederlandse samenvatting

Oxidatieprocessen geïnitieerd door enzymen zoals lipoxygenase en polyfenoloxidase vormen een belangrijke oorzaak van voedselbederf. Lipoxygenase is verantwoordelijk voor de oxidatie van meervoudig onverzadigde vetzuren wat leidt tot een vermindere smaak en het ranzig worden van vette voedingsmiddelen. Polyfenoloxidase is een initiator van bruinkleuring welke bijvoorbeeld plaatsvindt in groenten, fruit en champignons. Het gebruik van toevoegingen die in staat zijn om de oxidatieve processen te vertragen of te voorkomen (antioxidanten) is van belang in de conservering van voedingsmiddelen. Naast het bederf van voedingsmiddelen, spelen lipoxygenasen een rol in verschillende vormen van kanker, ontstekingsreacties, hart- en vaatziekten en neurodegeneratieve aandoeningen (Hoofdstuk 2). Polyfenoloxidasen zijn ook betrokken bij ziekten als kanker en neurodegeneratieve aandoeningen en zijn daarnaast verantwoordelijk voor pigment afwijkingen in de huid (Hoofdstuk 5). Het remmen van deze enzymen is dus niet alleen van belang in de voedingsmiddelenindustrie, maar ook in de farmaceutische en cosmetische industrie. Veel lipoxygenase en polyfenoloxidase remmers zijn inmiddels bekend. Hoewel deze stoffen vaak een sterke remming vertonen van de oxidatiereacties, zijn ze meestal niet in staat om het enzym zelf te deactiveren. De meeste remmers zijn polyfenolen die gewoonlijk hun werking ontlenen aan het afvangen van intermediaire radicalen of oxidatieproducten die gevormd worden tijdens de enzymreactie. Gedurende dit proces worden de remmers verbruikt en dit betekent dat hoge concentraties nodig zijn om oxidatie effectief tegen te gaan. Een directe remming van het enzym zelf is niet alleen efficiënter, maar ook een meer specifieke manier om ongewenste oxidatieve processen, zowel in voedingsmiddelen als in het menselijk lichaam, onder controle te houden. Vooral bij de toepassing als medicijn is deze specificiteit belangrijk om bijwerkingen te minimaliseren. Naast het gebrek aan specificiteit, zijn vele bekende antioxidanten synthetisch en/of toxisch en daarom niet toegestaan als additieven in voeding en cosmetische producten. De voorkeur voor natuurlijke antioxidanten heeft de zoektocht naar nieuwe verbindingen die in staat zijn om de nadelige oxidatiereacties tegen te gaan geïntensiveerd.

In de loop der jaren is gebleken dat eiwitten en peptiden uit diverse industriële bronnen een antioxidant werking vertonen in verscheidene oxidatiesystemen (Hoofdstuk 1). Het precieze werkingsmechanisme is echter onduidelijk. Eén belangrijk voordeel van eiwitten en peptiden
die uit natuurlijke bronnen zoals melk, ei, soja en tarwe worden afgeleid is dat ze over het algemeen als veilig worden beschouwd en daardoor in voedsel en cosmetische producten kunnen worden toegepast. Voorts kunnen eiwit-eiwit interacties zeer specifiek zijn en daarom zouden eiwitten en peptiden, door een directe interactie met het enzym, lipoxygenase en polyfenoloxidase kunnen remmen. Het doel van het onderzoek beschreven in dit proefschrift was het identificeren van peptiden als specifieke remmers voor lipoxygenase uit sojabonen (Glycine max) en tyrosinase, een polyfenoloxidase, uit champignons (Agaricus bisporus).

Een relatieve nieuwe strategie gebaseerd op maken van geïmmobiliseerde peptide bibliotheken met behulp van de SPOT synthese (Frank, 1992), werd gekozen voor de identificatie van peptiden die een interactie hebben met deze enzymen. Naast de ‘high-throughput’ screening van industrieel relevante peptiden verschaft deze aanpak de mogelijkheid om de enzym-peptide interactie in meer detail te bestuderen via substitutieanalyse. Na een initiële selectie werd de antioxidant activiteit van veelbelovende peptiden verder getest in verschillende oxidatiesystemen. Omdat twee verschillende enzymen zijn bestudeerd bestaat dit proefschrift uit twee delen, het eerste handelt over lipoxygenase remming en het tweede over tyrosinase remming.

Hoofdstuk 3 beschrijft de identificatie van melkeiwit peptiden die als remmer kunnen fungeren van lipoxygenase. Een peptide reeks bestaande uit overlappende peptiden afgeleid van de sequentie van β-caseïne werd gescreend op interactie met lipoxygenase-1 uit sojabonen (Schurink, 2006). Het bleek dat peptiden afkomstig uit vijf verschillende gebieden van de β-caseïne sequentie in staat zijn om te binden aan lipoxygenase-1. (Figuur 3.2). Gezien de aminozuursamenstelling en -volgorde, is er geen duidelijke correlatie tussen deze gebieden anders dan dat een lengte van acht aminozuurresiduen optimaal lijkt te zijn voor het binden. Eerdere geïsoleerde peptide remmers uit β-caseïne hydrolysaten (Rival, 2001a; Rival, 2001b) zijn gelokaliseerd binnen twee van de vijf gevonden gebieden. De drie nieuw gedetecteerde gebieden in β-caseïne geven duidelijk het voordeel aan van het gebruik van synthetische peptide bibliotheken. Een aantal lipoxygenase-1 bindende en niet-bindende peptiden zijn preparatief gesynthetiseerd en hun antioxidant activiteit is getest in diverse oxidatiesystemen. Verschillende β-caseïne peptiden bleken in staat om de lipoxygenase-1 reactie te remmen. Daarnaast bezitten enkele van deze peptiden, met name de tyrosine en tryptofaan bevattende peptiden, het vermogen om radicalen af te vangen. Dit is mogelijk een verklaring voor de sterke lipoxygenase-1 remming door deze peptiden. Enzym remming dat alleen te wijten is aan de interactie tussen lipoxygenase-1 en een peptide, werd waargenomen voor de peptiden SITRINKK en vooral RINKKIEK die beide afkomstig zijn uit
het N-terminale deel van β-caseïne. De kinetiek van de lipoxygenase-1 remming door RINKKIEK toonde aan dit peptide een niet-competitieve remmer is van lipoxygenase-1 met een $K_i$ tussen de 150-200 µM. Deze waarde ligt in dezelfde orde van grootte als gerapporteerde waarden voor bekende lipoxygenase remmers zoals ascorbinezuur, benzoëzuur, en bisfenol A. Om het belang van de aminozuurresiduen in RINKKIEK voor de interactie met lipoxygenase-1 te bepalen en de remming van het enzym mogelijk te verbeteren, werd een substitutie analyse uitgevoerd. De resultaten hiervan staan beschreven in Hoofdstuk 4. Een nieuwe peptide reeks bestaande uit verschillende RINKKIEK analogen en een aantal andere β-caseïne peptiden werd op cellulose membranen gesynthetiseerd. Er bleek een duidelijke relatie te bestaan tussen lipoxygenase-1 binding en de lading van een peptide. Een positieve lading van +4 bleek het meest optimaal te zijn (Figuur 4.4A). Verschillende andere experimenten toonden aan dat de positief geladen lysine residuen in RINKKIEK van belang zijn voor de lipoxygenase-RINKKIEK interactie. Substitutie van elk residu binnen RINKKIEK met één van de 19 andere natuurlijk voorkomende aminozuren toonde aan dat bijna elke vervanging een lagere lipoxygenase-1 binding tot gevolg heeft (Figuur 4.7). Dit suggereert dat niet alleen de lysine residuen, maar ook de andere residuen die aanwezig zijn in RINKKIEK van belang zijn voor de interactie met lipoxygenase-1. Nochtans vertonen peptiden waarbij de RINKKIEK aminozuurvolgorde door elkaar gehusseld is (bijvoorbeeld IRKEIKKN) geen beduidend lagere affiniteit voor lipoxygenase-1 en remmen ze het enzym in dezelfde mate als RINKKIEK zelf. Klaarblijkelijk is de aanwezigheid van deze residuen belangrijker dan hun positie in de peptide sequentie. Daarnaast wees een eiwitfluorescentiestudie op de aanwezigheid van meerdere bindingsplaatsen voor het peptide op het oppervlak van lipoxygenase-1 (Figuur 4.5). Beide waarnemingen impliceren dat de lipoxygenase-RINKKIEK interactie niet erg specifiek is. Desalniettemin bleken verbeteringen aan de sequentie van RINKKIEK mogelijk door vervanging van het negatief geladen glutaminezuur residu. Hoogstwaarschijnlijk zorgt het verkrijgen van een totale peptide lading van +4 ervoor dat de lipoxygenase-1 binding toeneemt (Figuur 4.7). Voor zowel RINKKIPK als RINKKISK gaat deze toename gepaard met een bijna drievoudige toename in lipoxygenase-1 remming (Tabel 7.1). De interactie tussen de geteste peptiden en lipoxygenase-1 is dus hoofdzakelijk gebaseerd op electrostatische interacties. De sterkte van de remming is desondanks beter dan van de bekende lipoxygenase remmer L-carnosine. Hoewel lastig vast te stellen door de complexiteit van de lipoxygenase reactie, resulteert de interactie tussen lipoxygenase-1 en de octapeptiden in een niet-competitieve remming zoals beschreven in Hoofdstukken 3 en 4.
Voor het onderzoek naar de remming van polyfenoloxidases door peptiden van industriële eiwitten werd tyrosinase uit champignons als modelenzym gekozen. Omdat er nog relatief weinig bekend was over dit remmingsmechanisme werden peptiden afkomstig uit diverse bronnen inclusief de melkeiwitten β-caseïne, α-lactalbumine en β-lactoglobuline, ovalbumine uit ei, gliadine uit tarwe en twee soja eiwitten glycinine and β-conglycinine onderzocht (Schurink, 2007). Naast de peptide bibliotheek gebaseerd op deze eiwitten, werd er ook een peptide reeks gemaakt en geanalyseerd met fragmenten van de fenoloxidase remmer uit de huisvlieg. De peptide bibliotheken werden gescreend op zowel tyrosinase binding als remming en de resultaten hiervan staan beschreven in Hoofdstuk 6. Sterke tyrosinase bindende peptiden bevatten altijd een arginine residu, vaak in combinatie met een fenylalanine residu. In tegenstelling tot de resultaten verkregen met het lipoxygenase, is er voor tyrosinase geen verband tussen peptide lading en enzym binding, hoewel een netto negatieve lading nadelig is voor de binding. Sterke tyrosinase remmende peptiden bevatten de hydrofobe residuen valine, alanine en/of leucine. Goede tyrosinase bindende en remmende peptiden, bijvoorbeeld LFRVASM uit ovalbumine (Tabel 7.1), hebben een combinatie van arginine en/of fenylalanine met valine, alanine en/of leucine in hun sequentie (Figuur 6.7). Het is mogelijk dat de hydrofobe residuen van deze peptiden het actieve centrum van tyrosinase, analoog aan de remmende domeinen beschreven voor verschillende type 3 koper eiwitten (Decker, 2007), kunnen afschermen. Meer experimenten zijn nodig om het precieze mechanisme van remming van tyrosinase door deze peptiden vast te stellen.

Op basis van de resultaten beschreven in dit proefschrift kan geconcludeerd worden dat de remming van de oxidatieve enzymen lipoxygenase en tyrosinase met behulp van peptiden haalbaar is. Hoewel de antioxidant capaciteit van deze peptiden in het zelfde bereik ligt als voor enkele algemene remmers, volstaan zowel de sterkte als de bedoelde specificiteit nog niet voor de meeste toepassingsdoeleinden. Aangezien de remming van de peptiden in deze studie is getoetst op model enzymen in *in vitro* oxidatiesystemen, moeten de resultaten als inleidend worden beschouwd. Extra studies zijn nodig om het effect en de stabiliteit van deze peptiden in meer complexere matrices zoals voeding en/of gehele celsystemen te bepalen. De aanwezigheid van andere componenten kunnen de enzym-peptide interactie verstoten, vooral wanneer de specificiteit laag is. Met betrekking tot de stabiliteit van de remmende peptiden, moet worden opgemerkt dat de remmers aan afbraak onderhevig zijn in aanwezigheid van proteases. Voor de productie van antioxidant peptiden echter, kunnen de opeenvolgende acties van proteasen of mengsels van proteasen bruikbaar zijn voor de
isolatie van actieve peptiden uit industriële eiwitten. Zo kunnen bijvoorbeeld verschillende RINKKIEK analogen van β-caseïne kunnen worden geïsoleerd met behulp van een aantal proteasen (Figuur 7.1).

De in dit proefschrift beschreven combinatie van SPOT synthese, ‘high-throughput’ screening, analyse van moleculaire determinanten en optimalisatie van enzym-remmer interactie is nog niet eerder gebruikt voor de identificatie van antioxidant peptiden. Hoewel vele peptiden met antioxidant activiteit in de literatuur zijn beschreven, is het voor het eerst dat de activiteit toegeschreven kan worden aan een directe interactie tussen enzym en peptide. Naast de ‘high-throughput’ methodologie, verschaft de SPOT synthese van peptide bibliotheken een krachtig hulpmiddel om de moleculaire interactie op een fundamenteel niveau te bestuderen. In combinatie met ‘quantitative structure-activity relationship’ (QSAR) (Collantes, 1995; Siebert, 2001), ‘in silico docking’ (Hetényi, 2002), 3D-modellering en/of co-kristallisatie experimenten kan dit leiden tot nieuwe inzichten met betrekking tot de optimale lengte, aminzuursamenstelling, sequentie en structuur van een antioxidant peptide. Wanneer deze zijn geïdentificeerd en verder gekarakteriseerd in geschikte (model)systemen, kunnen nieuwe technologische ontwikkelingen, zoals chromatografie op grote schaal, membraan technologieën en selectieve extractie/precipitatie procedures, mogelijkheden bieden voor de commerciële productie van antioxidant peptiden uit natuurlijke eiwitbronnen.

hebben peptiden de potentie om gebruikt te worden als geneesmiddel. Analisten schatten dat de wereldmarkt voor op peptide gebaseerde geneesmiddelen zich zal uitbreiden met een groeisnelheid die bijna het dubbele is van de groeisnelheid van geneesmiddelen in het algemeen (Marx, 2005). Deze ontwikkeling vereist specifieke methodes voor een betere evaluatie van de doelactiviteit, maar ook productieverbeteringen en betere stabilisatie- en toedieningstechnieken. Menselijk insuline bijvoorbeeld wordt nu geproduceerd voor algemeen klinisch gebruik via recombinant technieken (Shapley, 1982). In tegenstelling tot vele andere, niet op eiwit gebaseerde, geneesmiddelen kan insuline niet oraal worden ingenomen en wordt het gewoonlijk toegediend via injecties. Zoals bijna alle andere eiwitten die in het maagdarmkanaal worden geïntroduceerd, wordt insuline afgebroken tot korte eiwit fragmenten en vrije aminozuren, waarna alle bioactiviteit verloren gaat. Inkapselingsmethodes om bescherming en gecontroleerde afgifte van dergelijke bioactive peptiden in het menselijk lichaam te verzekeren kunnen dit verhelpen (Hildebrand, 2000; Reithmeier, 2001). De integratie van de in deze studie toegepaste methode voor het vinden en optimaliseren van bioactiviteit met nieuwe productie- en stabilisatiemethoden zal een nieuw platform kunnen verschaffen voor bioactieve peptiden in voeding en gezondheid.
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List of abbreviations

AA  arachidonic acid
AAPH 2,2'-azobis(2-amidinopropane) dihydrochloride
ABTS 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
Abu aminobutyric acid
BSA bovine serum albumin
CMC critical micelle concentration
DCT DOPAchrome tautomerase
DHCHBTA 2-dihydro-3-carboxy-5-hydroxy-1,4-benzothiazinyl-alanine
DHI 5,6-dihydroxyindole
DHCA 5,6-dihydroxyindole-2-carboxylic acid
DNA deoxyribonucleic acid
DOPA 3,4-dihydroxyphenylalanine
DPPH 1,1-diphenyl-2-picrylhydrazyl
EC Enzyme Commission
Fmoc 9-fluorenylmethoxycarbonyl
HBTA 5-hydroxy-1,4-benzothiazinylalanine
HPLC high pressure liquid chromatography
HSA human serum albumin
IC$_{50}$ inhibitory concentration 50%
ICAQ indole-2-carboxylic acid-5,6-quinone
IQ indole-5,6-quinone
LA linoleic acid
LMW low molecular weight
LOX lipoxigenase
LOX-MB Marina Blue® labeled lipoxigenase-1 from soybean
MB Marina Blue®
MBTH 3-methyl-2-benzothiazolinone hydrazone
MIP melanization-inhibiting protein
MS mass spectrometry
n.d. not determined
Nle norleucine
Nva norvaline
ORF open reading frame
PDB Protein Data Bank
PEG polyethylene glycol
POI phenoloxidase inhibitor
PPO polyphenol oxidase
PUFA polyunsaturated fatty acid
QSAR quantitative structure-activity relationship
ROS reactive oxygen species
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPPS solid-phase peptide synthesis
TFA trifluoroacetic acid
Tris tris-(hydroxymethyl) aminomethane
TRP tyrosinase related protein
TYR tyrosinase
TYR-MB Marina Blue® labeled tyrosinase from mushroom
UV ultraviolet
VIS visible
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![Chemical structure image](image-url)
**Curriculum vitae**

List of publications


Overview of completed training activities

**Discipline specific activities**

- International advanced course on ‘Protein engineering’, VLAG 2001
- International advanced course on ‘Chemistry and biochemistry of antioxidants’, VLAG 2001
- 3rd International advanced course on ‘Reaction kinetics in food science’, VLAG 2001
- Bioinformatics-1, Laboratory of Biochemistry, WUR 2002
- 2nd International advanced course on ‘Industrial proteins’, VLAG and IOP 2003

**General courses**

- PhD course ‘Guide to scientific artwork’, Microscopy L@b, WUR 2001
- 9th PhD week, VLAG 2002
- Organizing and supervising thesis work, OWU 2004

**Meetings of learned societies**

- Annual meeting of the study group ‘Protein Research’ in Lunteren 2001
- 10th ‘Van der Kerk’-lecture and mini-symposium ‘(Bio)organic synthesis and (bio)catalysis’, Utrecht University 2002
- ‘HLO Beroepensymposium’ organized by the KNCV at Organon in Oss 2002
- 10th Peptide day, University of Amsterdam 2003
- 4th International symposium on ‘Industrial proteins’ organized by IOP and VLAG in Ede 2003
- Annual meeting of the study groups ‘Design and Synthesis’, ‘Structure and Reactivity’ and ‘Biomolecular Chemistry’ in Lunteren 2003
- 2nd International conference on ‘Biocatalysis in the food and drinks industries’, Universität Hohenheim, Germany 2004
- Annual meeting of the study group ‘Protein research’ in Lunteren 2004
- 19th American peptide symposium ‘Understanding biology using peptides’ organized by the American Peptide Society in San Diego, USA 2005
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On the cover: Activity staining of mushroom tyrosinase binding to peptide spots on a cellulose membrane (Chapter 6).

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