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Natural compounds with potential antioxidant activity; *in vitro* study in a model of protein glycoxidation

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CONTENT

Publications closely related to the topic of presented doctoral thesis

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I. Držata, J., **Boušová, I.**, Maloň, P. (2005) Determination of quality of pyridoxal-5´-phosphate enzyme preparations by spectroscopic methods. J Pharm Biomed Anal. 37(5): 1173-1177. **p. 73**

II. **Boušová, I.**, Vukasović, D., Juretić, D., Palička, V., Držata, J. (2005) Enzyme activity and AGE formation in a model of glycoxidation of AST by D-fructose *in vitro*. Acta Pharm. 55: 107-114. **p. 79**

III. **Boušová, I.**, Martin, J., Jahodář, L., Dušek, J., Palička, V., Držata, J. (2005) Evaluation of *in vitro* effects of natural substances of plant origin using a model of protein glycoxidation. J Pharm Biomed Anal. 37(5): 957-962. **p. 88**

IV. **Boušová, I.**, Bakala, H., Chudáček, R., Palička, V., Držata, J. (2005) Glycation-induced inactivation of aspartate aminotransferase, effect of uric acid. Mol Cell Biochem. 278(1-2): 85-92. **p. 95**

V. Firuzi, O., Fuksa, L., Spadaro, C., **Boušová, I.**, Riccieri, V., Spadaro, A., Petrucci, R., Marrosu, G., Saso, L. (2006) Oxidative stress parameters in different systemic rheumatic diseases. J Pharm Pharmacol. 58(7): 951-957. **p. 104**

1. SUMMARY IN CZECH (ABSTRACT)

Reaktivní formy kyslíku a dusíku (RONS) a volné radikály se staly v posledním desetiletí významným předmětem studia v mnoha oborech včetně medicínských. Stav, při němž je rovnováha mezi vznikem a odbouráváním volných radikálů v organismu posunuta ve prospěch jejich vzniku, se nazývá oxidační stres. Při nadbytku volných radikálů v organismu dochází k poškození biomolekul a tkání, což může vést až ke vzniku onemocnění. Oxidační stres se podílí na vzniku celé řady onemocnění, např. diabetu mellitu, aterosklerózy, revmatoidní artritidy, Alzheimerovy choroby a dalších, ale také na fyziologickém procesu stárnutí. Ve snaze pozastavit průběh stárnutí a odvrátit vznik onemocnění se začala používat různá potravní aditiva s obsahem vitamínů a antioxidantů přírodního původu.

Diabetes mellitus je onemocnění charakterizované hyperglykémií a je vždy provázeno oxidačním stresem. Glukóza a různé intracelulární cukry (fruktóza, ribóza, glyceraldehyd a další) se kovalentně váží na volné aminoskupiny proteinů a způsobují poškození jejich struktury. Tento proces se nazývá neenzymová glykace. Sledem následných reakcí vznikají pozdní produkty pokročilé glykace (AGEs), které se podílí na tvorbě kovalentních vazeb mezi sousedními molekulami proteinů (crosslinks). Mezi nejvýznamnější AGEs patří N-ε-karboxymetyllysin, pyrralin, pentosidin a argpyrimidin. Během autooxidace glykujícího cukru a AGEs se tvoří volné radikály a různé reaktivní meziprodukty (např. α-dikarbonylové sloučeniny). Ty se dále podílejí na rozvoji neenzymové glykace. Protože je tato doprovázena oxidačními procesy, které jsou často katalyzované ionty přechodných kovů, lze celý proces označit jako glykoxidaci.

Jedním z cílů této práce, která navazuje na několik předešlých diplomových prací, bylo zavést a optimalizovat *in vitro* model glykace aspartátaminotransferasy (AST). Optimalizace začala zvolením vhodného, komerčně dostupného preparátu AST a pokračovala výběrem dostatečně účinného glykačního činidla ve vhodné koncentraci. Pro sledování průběhu glykace bylo vhodné rozšířit spektrum používaných metod o metody ke stanovení množství vznikajících produktů, např. fluorescenční. V zavedeném modelu glykace se sledovala jednak přímá interakce jednotlivých antioxidantů s molekulou proteinu a také vliv antioxidantů na glykaci proteinu.

Za použití spektroskopických a fluorimetrických metod byl jako nejvhodnější zdroj AST pro další experimenty zvolen preparát firmy Serva, který vykazoval všechny v literatuře popsané spektrální charakteristiky AST a měl také nejmenší počáteční koncentraci fluorescenčních AGEs. Ve prvním modelu glykace byla jako glykační

agens použita 50 milimolární D-fruktóza, která patří mezi významné intracelulární cukry se značnou reaktivitou. Ve snaze podpořit oxidační procesy byly do tohoto modelu přidány ještě ionty mědi a železa, které katalyzují radikálové reakce. Vhodnou koncentrací těchto iontů se zdá být 1 μM koncentrace. Kvůli dlouhému trvání jednoho experimentu (až 21 dnů) jsme postupně tento model opustili a v současné době provádíme optimalizaci modelu, kde je jako glykačního agens použito metylglyoxalu, což je vysoce reaktivní dikarbonylová sloučenina, která vzniká jako meziprodukt neenzymové glykace.

Pro stanovení množství vznikajících AGE produktů byla zavedena fluorescenční metoda k měření množství jak celkových fluorescenčních AGEs tak pouze pentosidinu. Měření probíhá při specifických vlnových délkách excitace a emise, které odpovídají celkovým AGEs ($\lambda_{exc}/\lambda_{em}$ 370/440 nm) a pentosidinu ($\lambda_{exc}/\lambda_{em}$ 335/385 nm). Metody pro stanovení parametrů oxidačního stresu u pacientů s revmatoidní artritidou by se daly s výhodou použít i ke sledování průběhu glykace v našem modelu.

Antioxidanty přírodního původu, které byly testovány v rámci této doktorské práce, vykazovaly jak pozitivní antiglykační účinky, tak negativní přímé účinky na aktivitu AST a to v různém rozsahu. Mezi látky, které měly jednak nejlepší antiglykační vlastnosti a také nejméně ovlivňovaly aktivitu samotného modelového proteinu, patřily kyseliny hydroxycitronová, *o*-kumarová a močová. Naopak látkami s celkovým negativním projevem byly flavonoid bajkalin a jeho aglykon bajkalein a metylarbutin. Tyto látky inhibovaly aktivitu AST a nikterak nezabránily její glykaci. Dalo by se říci, že antioxidanty rostlinného původu s výraznými zhášecími schopnostmi bývají také nejreaktivnějšími látkami, co se interakce s molekulou proteinu týče.

2. INTRODUCTION

This doctoral thesis has been elaborated at the Department of Biochemical Sciences of the Faculty of Pharmacy in Hradec Králové, Charles University. The research team of this department has a long-standing experience with the study of non-enzymatic glycation of enzymes *in vitro*. This topic is highly up-to-date in the context of current understanding of mechanisms accompanying diabetes mellitus and the process of aging. Nowadays, much attention is paid to the research of protein glycation. Several diploma theses dealing with this topic, including my own, have been elaborated at our department. Existing cooperation with the *Laboratoire de Biologie et Biochimie Cellulaire du Vieillissement, Université Paris 7 – Denis Diderot* in the framework of Erasmus program provides a possibility for our students to elaborate their diploma theses in this laboratory. A few scientific articles have been published in national as well as in international journals by this research group (Beranek et al. 2001; Beranek et al. 2002; Beranek et al. 2006; Drsata et al. 2002).

Purified aspartate aminotransferase (AST) from the porcine heart has been chosen as the model protein based on previous results of our research team. This enzyme is commercially available in highly purified, stable form. Several monosaccharides (e.g. glucose, ribose and glyceraldehyde) were tested and they exerted either too low (glucose) or too high glycation activity (glyceraldehyde) (Beranek et al. 2002; Drsata et al. 2002). Preliminary experiments testing potential antiglycation activity of natural antioxidants were accomplished. Enzyme activities, which served as a measure of proceeding protein glycation in previous experiments, were assayed by two methods: UV kinetic and dinitrophenylhydrazine. Measurements using UV kinetic method were performed in cooperation with the Institute of Clinical Biochemistry and Diagnostics, Faculty Hospital in Hradec Králové.

The presented work was designed with a view to gain new pieces of knowledge about protein glycation and a possible intervention of antioxidants into this process. The main goals of the project were to establish and optimize a suitable model of *in vitro* glycation of aspartate aminotransferase as well as to introduce fluorescent methods for determination of advance glycation end-products formation. The following step in the project was to study the influence of natural antioxidants. Direct interactions of individual antioxidants with the molecule of protein as well as possible antiglycation effect of those antioxidants were studied. Structure-activity relationships in the group of phenolic antioxidants were observed.

3. GENERAL INTRODUCTION

Oxidative stress

Generation of reactive oxygen and nitrogen species (RONS) and their scavenging by antioxidative system of an organism are in approximate balance in healthy subjects. The state in which this fragile equilibrium is seriously impaired is called “oxidative stress” (Sies and Cadenas 1985). The cause of this imbalance is either an excessive generation of free radicals or an insufficient activity of the antioxidant defense systems of the organism (or the combination of both processes). Oxidative stress can lead either to cellular adaptation (up-regulation of the defense system components), to tissue injury (all types of biomolecules), or to cell death caused by apoptosis or necrosis (Davies 1999; Lu et al. 1993).

Excessive generation of RONS and thus oxidative stress plays an important role in the pathogenesis of major part (if not in all) of human diseases. Oxidative stress is not always the main cause of the disease initiation, but usually occurs as a consequence of a previous tissue damage, e.g. infection, toxins or trauma. The main cause of the disease appearance can be also ionizing radiation (generation of hydroxyl radicals, which impairs protein, DNA, and lipid molecules) or deficit of antioxidants (Halliwell 1994; Halliwell and Gutteridge 1999). Among the diseases with significant contribution of oxidative impairment are diabetes mellitus, atherosclerosis, Alzheimer’s disease, rheumatoid arthritis and many others. Oxidative stress plays an important role also in the physiological process of aging (Davies 1995).

3.1.1. Reactive oxygen and nitrogen species (RONS)

Free radicals can be defined as any chemical species, which contain minimally one atom orbital with one unpaired electron in its outermost shell (O_2^{-}•, HO•, NO•). Reactive oxygen and nitrogen species (RONS) is a general term for both free radicals and non-radical compounds participating in their metabolism, which do not have the unpaired electron in outer valence shell (HClO, H_2O_2, $ONOO^{-}$). Free radicals possess extremely unstable configuration and therefore they rapidly react with other molecules or radicals to achieve the stable configuration of electrons in their valence shell (Betteridge 2000; Halliwell and Gutteridge 1999). Several examples of RONS with their approximate biological half-lives are mentioned below in Table 1.

Free radicals can be generated either by homolytic fission, by reduction or by oxidation of a neutral molecule. Singlet oxygen ($^1O_2$) is formed by spin inversion from

ground state oxygen upon absorption of electromagnetic radiation. Free radical formation is often the first step (so called initiation) of a radical chain reaction, which can be the main cause of tissue injury (Štípek 2000).

Table 1. Reactive oxygen and nitrogen species and their biological half-lives. Table has been modified according to Štípek (2000).

Free radicals		**Non-radicals**	
(A) Reactive oxygen species (ROS)			
Superoxide anion, $O_2^{-}\bullet$	2-4 μs	Hydrogen peroxide, H_2O_2	1 ms
Hydroxyl radical, HO•	1-10 ns	Hypochlorous acid, HClO	
Peroxyl radical, ROO•	7 s	Hypochlorite, ClO^{-}	
Alkoxyl radical, RO•	10^{-6} s	Ozone, O_3	
Hydroperoxyl radical, $HO_2\bullet$	>1 s	Singlet oxygen, $^1O_2$	2-7 μs
(B) Reactive nitrogen species (RNS)			
Nitric oxide, NO•	1-10 s	Nitrosyl, NO^{+}	
Nitrogen dioxide, $NO_2\bullet$		Nitrous acid, HNO_2	
		Peroxynitrite, $OONO^{-}$	1s
		Alkylperoxynitrite, $ROONO^{-}$	

The variable oxidation number of transition metal ions (e.g. Cu, Fe) helps them in effective catalysis of redox reactions and they are used for this purpose at the active sites of many enzymes. The potential danger is in the possibility of these ions to catalyze also unwanted free-radical reactions. A prime example of such a deleterious process catalyzed by transition metal ions is Fenton chemistry (oxidation of organic compounds in the system H_2O_2/Fe). The reaction can be described as it is written below although the exact mechanism of the reaction is not fully understood. However, $O_2^{-}\bullet$ can act as the reductant to re-reduce Fe^{3+} to Fe^{2+} in the so-called Haber-Weiss reaction. It should be noted that copper can substitute iron in both reactions (Davies 1995; Halliwell and Gutteridge 1999). The overall reaction of RONS interchange can be described as follows:

Fenton reaction (1894)	$H_2O_2 + Fe^{2+} \rightarrow HO\bullet + HO^{-} + Fe^{3+}$
Haber-Weiss reaction (1934)	$O_2^{-}\bullet + Fe^{3+} \rightarrow O_2 + Fe^{2+}$
Overall reaction	$H_2O_2 + O_2^{-}\bullet \rightarrow O_2 + HO\bullet + HO^{-}$

Reactive oxygen and nitrogen species possess not only deleterious properties but also several important physiological roles. Hydrogen peroxide is essential for the synthesis of triiodothyronine and thyroxine in thyroid gland (Igo et al. 1964). Phagocytic cells generate RONS (O_2^{-}•, H_2O_2, NO•, HOCl, and $OONO^{-}$) in order to kill invading organisms (Babior 2000). Free radicals are used by several enzymes, e.g. cytochrome oxidase in respiratory chain (Turrens 2003) and monooxygenases during hydroxylation of endogenous as well as exogenous substrates (Štípek 2000). RONS act as signaling molecules in the redox-signaling pathways via the transcription factors NF-κB and AP-1 (Remacle et al. 1995). Nitric oxide is generally accepted as one of the most important signaling molecules (Pfeilschifter et al. 2003). Nitric oxide acts as neurotransmitter in CNS and stimulates the synthesis of some other neurotransmitters (glutamate, noradrenalin), causes relaxation of sphincters and acts as an important vascular dilator also called EDRF (endothelium-derived relaxing factor).

3.1.2. Molecular impairment caused by RONS

Reactive oxygen and nitrogen species can also exert toxic properties under certain circumstances and attack virtually all biomolecules. The most frequent targets of free radicals are biomolecules of vital importance such as proteins, lipids and DNA. The mechanism of their impairment by RONS, its consequences and possibilities of repair are described below.

3.1.2.1. Damage to lipids: lipid peroxidation and cholesterol oxidation

Lipid peroxidation has been extensively studied over the past decades. The initiation of peroxidation sequence is the attack of ROS, which can abstract a hydrogen atom from a methylene group of a lipid molecule. Unsaturated fatty acids are prone to free-radical attack. The resulting radical tends to be stabilized by a molecular rearrangement to form a conjugated diene, which is able to combine with molecular oxygen to give peroxyl radical. The peroxyl radical is capable of abstracting H atom from the adjacent fatty acid, especially in the presence of Cu or Fe ions, thus causing an autocatalytic chain reaction (propagation stage). The peroxyl radical combines with H atom to form lipid hydroperoxide. Alternatively, peroxyl radicals can be transformed into cyclic peroxides or cyclic endoperoxides. The radical reaction is terminated after meeting with another radical or chain-breaking antioxidant (α-tocopherol). Lipid peroxides are relatively stable compounds, but they can decompose into alkoxyl and

peroxyl radicals in transition metal catalyzed reactions. These newly generated compounds can stimulate further lipid peroxidation and can also be further decomposed into toxic aldehydes (malondialdehyde, 4-hydroxynonenal) and hydrocarbons with short chain (ethane, pentane). Some cross-bonds between MDA (malondialdehyde) or 4-HNE (4-hydroxy-2-nonenal) and proteins are also possible (Halliwell and Gutteridge 1999; Štípek 2000).

Cholesterol in membranes and lipoproteins can be oxidized during lipid peroxidation giving a mixture of oxidized products. Several examples of the arising oxysterols are cholesterol-7-hydroperoxides; cholest-5-ene-3-β7-diols; 3β-hydroxycholest-5-en-7-one; 5,6β(α)-epoxy-5β-cholestan-3-β-ols and 5α-cholestane-3β,5,6β-triol. The cytotoxicity of these products remains unclear, because conflicting reports were published (Brown and Jessup 1999; Sevanian and Peterson 1986).

Extensive peroxidation in cell membranes leads to changes in fluidity, increased permeability, decreased membrane potential, and eventually to membrane rupture (Stark 2005). Peroxides within membranes can be converted into appropriate alcohols by phospholipid hydroperoxide glutathione peroxidase enzymes or may be cleaved from the membrane by the action of phospholipases.

3.1.2.2. Damage to proteins: protein oxidation and nitration

Damage to proteins can occur either by direct attack of RONS upon them or by secondary impairment involving attack by lipid peroxidation end products (e.g. MDA, 4-HNE). Individual amino-acid residues can be attacked by different ROS and form various oxidation products, e.g. methionine is oxidized into methionine sulfoxide and sulfone, cysteine to cysteine disulfide and further to sulfenic acid, histidine to 2-oxohistidine, and tryptophan to kynurenine. Arginine and prolin form glutamic semialdehyde upon oxidation. Tryptophan, phenylalanine and tyrosine are also nitrated by peroxynitrite to 6-nitrotryptophan and 3-nitrotyrosine (Stadtman and Levine 2003).

Many proteins can sustain considerable damage without impairment of their physiological functions. For enzymes, the damage will become evident in activity measurements only when the essential amino acid residue at or close to the active sites are impaired. Not only enzymes but also receptors and transport proteins can be important targets of free-radical damage, which can lead to loss of their physiological functions. Other important consequences of protein oxidation are formation of new

antigenic determinants with subsequent autoimmune reaction and disturbed homeostasis of the cells upon changes in ionic pump activity (Halliwell and Gutteridge 1999; Štípek 2000). Glutathione or thioredoxin can re-reduce disulfide bridges formed by cysteine SH groups oxidation. Another important repair system is peptide methionine sulfoxide reductase, which can re-activate proteins previously oxidized at their Met residues (Moskovitz et al. 1999). Oxidized proteins are often marked by ubiquitin *in vivo* and then degraded by proteolytic enzymes. These proteases often occur in large multienzyme complexes called proteasomes (Hilt and Wolf 2004).

3.1.2.3. Damage to DNA: strand breakage and bases impairment

DNA itself is a very stable molecule, but oxidative stress highly accelerates its damage. Multiple products are generated from DNA upon exposure to HO•, since it attacks sugars, purines and pyrimidines. The interaction of guanine base with HO• leads to the formation of 8-hydroxy-7,8-dihydro-2'-deoxyguanosine; 8-hydroxyguanine and 2,6-diamino-5-formamido-4-hydroxypyrimidine after opening of the imidazole ring. Adenine reacts with hydroxyl radical in a similar manner to guanine. Pyrimidine bases are also subjected to ROS attack giving multiple oxidation products such as thymine glycol, uracil glycol, cytosine glycol, 5-hydroxy-5-methylhydantoin, 5-hydroxy-6-hydrothymine and many others. The deoxyribose sugar moiety is oxidized and fragmented by HO•, which causes free bases release, DNA strand breakage, and MDA generation (Cadet et al. 2003; Valko et al. 2004).

DNA damage can result in single or double strand breakage and cross-link formation with adjacent DNA or protein molecule. If the lesions are not repaired, subsequent DNA replication can lead to mutations or apoptosis. Mutations in genetic information can further initiate carcinogenesis and aging (Štípek 2000). Some examples of direct removal of unwanted changes from DNA are DNA photolyase enzyme and O^6-methylguanine-DNA-methyltransferase. In general, mispaired, oxidized and deaminated bases are removed from DNA either by nucleotide excision repair mechanism (excinuclease complex) or by base excision repair mechanism (DNA glycosylase). The repair of double-strand breaks is mediated by multiple proteins, e.g. DNA-dependent serine/threonine kinase (Halliwell and Gutteridge 1999; Jackson 1997; Sancar 1996).

3.1.3. Free radical diseases

RONS may cause a significant contribution to the disease pathology at one time; at other times they may not. Although oxidative stress may often be a consequence of the tissue damage, it does play an important role in furthering tissue injury in several important human diseases. Diabetes mellitus, atherosclerosis, chronic inflammatory diseases, ischemia-reperfusion injury, neurodegenerative diseases, and malignant diseases are important examples of the so-called “free radical diseases”.

3.1.3.1. Diabetes mellitus

Diabetes mellitus is a chronic metabolic disorder characterized by increased levels of blood glucose (hyperglycemia) and urinary glucose excretion. Diabetes is caused either by faulty production of insulin (juvenile onset, type I, IDDM) or by poor tissue response to the available insulin (type II, NIDDM). This disease is always accompanied by oxidative stress. There is a markedly increased formation of reactive oxygen species and lipoperoxides as well as altered levels of antioxidants (glutathion, ascorbic acid, α-tocopherol, lipooic acid) and antioxidant enzymes (catalase, superoxid dismutase) in the blood and tissues of the diabetic patients (West 2000). Complications of poorly controlled diabetes include macrovascular disease (accelerated atherosclerosis) and microvascular diseases of the eye (diabetic retinopathy), kidney (diabetic nephropathy), and peripheral nerves (diabetic neuropathy). Retina, kidneys, nerves, and eye-lens are insulin-independent tissues, which are freely permeable for glucose and so suffer the most.

Hyperglycemia is a hallmark of both types of diabetes and at the same time the main cause of the development of chronic complications. Glucose in its opened form covalently binds to amino groups of proteins, phospholipids, and nucleic acids. This process is called non-enzymatic glycation. Covalent cross-links between adjacent modified protein chains are formed (Monnier 1989). Superoxide and other ROS (H_2O_2, HO•) are generated during autoxidation of glucose and advanced glycation end-products (AGEs). Glycation is thus accompanied by oxidation and the whole process can be designed as glycoxidation (Baynes 1991). Glycoxidation reactions are often catalyzed by transition metal ions, which in consequence enhance the rate of oxidative stress. Non-enzymatic glycation is described in detail in chapter 3.3.

Glycation of collagen causes a decrease in elasticity of connective tissue and renal basal membrane injury. Glycated apoB in LDL is not recognized by LDL scavenger receptor and it becomes a target for macrophages, which leads to foam cell formation and acceleration of atherosclerosis in diabetics (Press and Wilding 1989). Also some antioxidant enzymes can be modified by glycation, e.g. Cu,Zn-superoxide dismutase is inactivated and fragmented by glycation (Yan and Harding 1997).

3.1.3.2. Atherosclerosis

Atherosclerosis is a disease of large and medium-sized muscular arteries and it is characterized by endothelial dysfunction, vascular inflammation, and the buildup of lipids, cholesterol, calcium, and cellular debris within the intima of the vessel wall. In general, three types of thickening are recognized: fatty streaks (presence of foam cells), fibrous plaques and complicated plaques. Plaque formation can lead to vascular remodeling, acute and chronic luminal obstruction, abnormalities of blood flow and diminished oxygen supply to target organs resulting in myocardial infarction, stroke or lower-limb ischemia (Murray et al. 2003).

Activated monocytes and macrophages produce RONS in the vessel wall, which may injure neighboring cells. Increased plasma levels of total cholesterol and LDL are important risk factors of atherosclerosis. LDL particles penetrating into the vessel wall are oxidized and further taken up by the scavenger receptor of macrophages. Lipid-laden macrophages are changed into foam cells (Halliwell and Gutteridge 1999; Štípek 2000). Oxidized lipids may degrade to reactive carbonyl compounds that react with proteins, forming advanced lipoxidation end-products (ALEs). As it was mentioned above, LDL is also changed by glycation reactions forming AGEs (Baynes and Thorpe 2000). Increased plasma level of homocysteine is another important risk factor of atherosclerosis. Although the exact mechanism of its vascular toxicity remains unknown, homocysteine can be oxidized *in vitro* in the presence of transition metal ions and $O_2^{-}\bullet$, H_2O_2, HO•, and sulfur radicals are formed (Dudman et al. 1993).

3.1.3.3. Neurodegenerative diseases

Parkinson’s disease, Alzheimer’s disease, amyotrophic lateral sclerosis (Barnham et al. 2004), multiple sclerosis (Gilgun-Sherki et al. 2004), and Friedreich's ataxia (Calabrese et al. 2005) represent just a few examples of the neurodegenerative diseases, in which pathogenesis oxidative stress plays an important role. The mentioned

diseases are not subject of this thesis and therefore only some general conditions leading to the nervous system damage are mentioned below.

The brain is especially sensitive to oxidative damage. One of the reasons can be its high O_2 consumption per unit of tissue mass. High content of polyunsaturated fatty acids (PUFA) is present in the neuronal membrane lipids. PUFA are especially prone to lipid peroxidation. Microglia, resident macrophages of the nervous system, are able to produce O_2^{-}•, H_2O_2 and probably also NO• upon activation. Mitochondria in the brain can produce O_2^{-}•, which may cause mitochondrial DNA damage. Furthermore, many neurotransmitters (dopamine, L-DOPA, and noradrenalin) are autoxidizable molecules and RONS are generated during their oxidation. The cerebrospinal fluid has a very low capacity to bind the released iron, which is capable of catalyzing free radical reactions. Moreover, antioxidant defenses in the brain are modest and especially the level of catalase is low. The main antioxidants present in the brain are glutathione, ascorbate, α-tocopherol, carnosine, metal-binding proteins, superoxide dismutase, and glutathione peroxidase (Halliwell and Gutteridge 1999).

3.2. Antioxidant defenses against oxidative stress

Living organisms are constantly exposed to a variety of pro-oxidant agents, which are able to damage vitally important biomolecules. In order to survive, organisms were forced to develop defense mechanisms, which would protect them against pro-oxidant agents, oxidative stress and the following cellular and tissue damage (Davies 1995). The three self-defense mechanisms are prevention of RONS formation, reparation of the impaired molecules, and scavenging and elimination of already formed free radicals by the antioxidant enzymes and low-molecular antioxidants. The composition of antioxidant defenses differs from tissue to tissue and it is not homogenous even in the different cell-types of the same tissue.

Although there has been a remarkable increase in scientific articles dealing with antioxidants and oxidative stress in recent years, the definition of the term antioxidant is still not clear. Halliwell (1990) has defined an antioxidant as any substance that, when present at low concentrations compared with those of the oxidizable substrate, considerably delays or inhibits the oxidation of the substrate. The term "oxidizable substrate" means every type of molecule found *in vivo*, e.g. proteins, lipids, DNA, and saccharides (Halliwell and Gutteridge 1999).

Several criteria exist for classification of antioxidants. These compounds can be divided according to their origin (endogenous x exogenous), solubility (hydrophilic x lipophilic), cellular localization, molecular weight, mechanism of action or the type of free radical the antioxidant interacts with (Štípek 2000). Antioxidants are made up of three different classes (see also Table 2 below), when the classification according to their molecular weight is applied:

- Antioxidant enzymes (e.g. superoxide dismutase, catalase, and glutathion peroxidase)
- Metal sequestering proteins (e.g. ceruloplasmin, ferritin, haptoglobins, and haemopexin)
- Low-molecular antioxidants (e.g. vitamins C and E, α-lipoic acid, and uric acid)

Table 2. Classification of important physiological antioxidant defense systems mentioned in the text below.

Antioxidant enzymes	Non-enzymatic antioxidants	
	Hydrophilic	Lipophilic
Superoxide dismutase	Ascorbic acid	Carotenoids
Catalase	Glutathione	Tocopherols
Glutathione peroxidase	Uric acid	Coenzyme Q
Glutathione reductase	Albumin	Flavonoids
Peroxiredoxine	Ceruloplasmin	Resveratrol
	Transferrin	

3.2.1. Antioxidant enzymes

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx), and peroxiredoxins are considered to be the most important antioxidant enzymes.

Superoxide dismutase catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide (equation 1) (Tainer et al. 1983). Hydrogen peroxide generated by SOD is decomposed to ground-state O_2 in the reaction catalyzed by **catalase** (equation 2). CAT can also catalyze peroxidase reactions (equation 3). Both enzymes interact directly with ROS species unlike GPx and peroxiredoxines (Halliwell and Gutteridge 1999).

Equation 1: $\mathbf{O_2^{-}\bullet + O_2^{-}\bullet + 2\ H^{+} \rightarrow H_2O_2 + O_2}$

Equation 2: $\mathbf{2\ H_2O_2 \rightarrow 2\ H_2O + O_2}$

Equation 3: $\mathbf{H_2O_2 + ROOH \rightarrow H_2O + ROH + O_2}$

Glutathione peroxidase and **peroxiredoxin 6** remove hydrogen peroxide by coupling its reduction with oxidation of reduced glutathione (GSH). The scheme of the reaction is mentioned below (equation 4). GPx and peroxiredoxine 6 are able to decompose fatty acid hydroperoxides (equation 5). These hydroperoxides must first be released from lipids by phosholipase A_2 in the case of GPx (Maiorino et al. 1991). Peroxiredoxine 6 is a so-called moonlighting enzyme, which posses both antioxidant and phospholipase A_2 activity (Manevich et al. 2004). The conversion of oxidized glutathione (GSSG) back to GSH is achieved by **glutathione reductase** (Bellomo et al. 1987), which catalyzes the reaction mentioned below in equation 6.

Equation 4: **$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$**

Equation 5: **$2\ GSH + ROOH \rightarrow GSSG + H_2O + ROH$**

Equation 6: **$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$**

3.2.2. Metal sequestering proteins

Some plasmatic proteins possess the ability to bind transition metal ions (Cu and Fe), change their oxidation status, and hence prevent their contribution to radical chain reactions.

Transferrin and **lactoferrin** belong to the group of proteins, which are capable of tight but reversible binding of two iron ions in the form of Fe(III). This iron is in transfer form and cannot catalyze Fenton's reaction (Huebers et al. 1981). Proteins responsible for storage of iron ions are **ferritin, hemosiderin,** and **neuromelanin**. Ferritin keeps iron ions in the Fe(III) redox state until it is released by a substance with strong reducing properties e.g. ascorbic acid (Harrison and Arosio 1996). **Haptoglobin** and **haemopexin** bind extracellular haemoglobin and haem, respectively (Gutteridge 1987; Gutteridge and Smith 1988).

Ceruloplasmin is a plasma protein responsible for binding more than 90% of circulating copper. Moreover, this protein has ferroxidase activity (oxidation of Fe(II) to Fe(III)), which is important in the mobilization of iron storage in the cells (Gutteridge 1978). Another protein capable of binding copper is **albumin** (Marx and Chevion 1986).

3.2.3. Low-molecular antioxidants

Packer (1995) has modified Halliwell's definition of antioxidant (Halliwell 1990) and has pointed out several criteria for evaluation of the antioxidant potential of a compound. This definition is appropriate for low-molecular antioxidants, but does not take into account the ability of antioxidant enzymes and metal sequestering proteins to act as antioxidants. Some of those criteria referring to chemical, biochemical, and pharmacological properties of the tested substance are mentioned below. An "ideal" antioxidant should: be absorbed from the diet and be available after absorption; be converted to its reduced form in cells and tissues so it can act as a proton or electron donor; be able to destroy free radicals and exhibit a variety of antioxidant actions, including interaction with other antioxidants in membrane and aqueous phases; and it

should be nontoxic. Antioxidants exert their antioxidant properties by several mechanisms:

- scavenging reactive oxygen and nitrogen species
- chelating transition metal ions (Cu, Fe)
- inhibiting the activities of enzymes involved in RONS generation (iNOS)
- interacting with other components of the antioxidant system

A list of some of the most important low-molecular antioxidants is mentioned below. The group of plant phenols (flavonoids, hydroxycinnamic acids, resveratrol) is described later in detail.

Ascorbic acid is a versatile, water-soluble antioxidant, and a cofactor of enzymes involved in the post-translational modification of collagen (prolyl and lysyl hydroxylases) and in the metabolism of dopamine (dopamine-β-hydroxylase). This compound is also important for the absorption of iron from the diet and its later mobilization from ferritin storage (Harrison and Arosio 1996; Hungerford and Linder 1983). It can be synthesized from glucose in the liver by most animals except primates including humans, guinea pigs, bats, and some birds, and for these it is obtained via the diet (Dewick 2002). Ascorbate reduces inorganic as well as organic free radicals, e.g. superoxide, hydroxyl radical, alkoxyl radical, peroxynitrite, and interacts also with hypochlorous acid and singlet oxygen (Bodannes and Chan 1979; Carr et al. 2000; Rose and Bode 1993). It may regenerate α-tocopherol from α-tocopheroxyl (or α-tocopheryl) radical by donating an hydrogen atom (May et al. 1998). The generated ascorbyl radical may be converted back to ascorbic acid either in a non-enzymatic reaction using GSH or in an enzyme-dependent reaction using NADH, NADPH or GSH (Coassin et al. 1991). Under certain circumstances, ascorbic acid may act not only as an antioxidant but also as a pro-oxidant. It is capable of reducing Fe(III) to Fe(II) that can then participate in the Fenton reaction (Burkitt and Gilbert 1990).

The tripeptide γ-glutamyl-cysteinyl-glycine i.e. glutathione (GSH) is one of the most ubiquitous peptide found in cells. It contains an unusual peptide linkage between the amino group of cysteine and the carboxyl group of the glutamate side chain. GSH is involved in detoxification, transport, metabolism, and in protection of the organism against some pro-oxidants (e.g. H_2O_2 and lipid hydroperoxides). For instance,

glutathione transports amino acids across the membrane; it is also involved in synthesis of leukotriene, and it participates in the regulation of cellular metabolism. GSH also acts as a cofactor for a number of enzymes (e.g. GPx, glutathione S-transferase, and Prdx6) and can serve as a storage form for cysteine (Voet and Voet 2004). The main function of GSH in the cells is protection of proteins containing sulfhydryl-groups (-SH) against oxidation, because those –SH groups are essential for their correct function (Cardoso et al. 1998).

Carotenoids (tetraterpenes) are a class of natural lipid-soluble pigments found mainly in plants, where they play a role in photosynthesis. The most important representatives of this group are β-carotene, lycopene, lutein, and zeaxanthin. Vitamin A (retinol), which is further converted into *cis*-retinal essential to the process of vision, is formed by cleavage of the molecule of β-carotene in mucosal cells of intestine. The compound with the most pronounced antioxidant activity among this group is lycopene. In contrast, vitamin A possesses almost no antioxidant activity. Carotenoids are capable of singlet oxygen quenching, reduction of carbon-centered radicals, and lipid alkylperoxides. They probably act in cooperation with tocopherols or other antioxidants. Several epidemiological studies have been performed on the effect of β-carotene in prevention of carcinogenesis, but the results of those studies are not explicit. ABTC (Rautalahti et al. 1997) and CARET studies (Goodman et al. 2004) showed an increase in the incidence of pulmonary carcinoma in smokers supplemented by β-carotene, while the study of Correa et al. (2000) suggests a beneficial role of β-carotene supplementation in retarding progression of premalignant gastric lesions.

Probably the most important lipid-soluble antioxidant is α-tocopherol (vitamin E). It is a so-called "chain-breaking" antioxidant, which effectively inhibits lipid peroxidation in biomembranes and in lipoprotein particles in plasma (Niki et al. 1989). α-Tocopheroxyl radical is generated during its antioxidant activity. It lacks the antioxidant activity of the parent molecule and can act as oxidant under certain circumstances. α-Tocopherol is regenerated in cooperation with other low-molecular antioxidants, e.g. ascorbic acid and flavonoids (Amorati et al. 2002).

Coenzyme Q_{10} (ubiquinol), the derivative of benzoquinol with 10 isoprene subunits in the side chain, is a component of the mitochondrial respiratory chain (in oxidized form, i.e. ubiquinon) and also works as an antioxidant. It protects efficiently

not only membrane phospholipids from peroxidation but also mitochondrial DNA and membrane proteins from free-radical-induced oxidative damage. It is capable of regenerating α-tocopherol (Pobezhimova and Voinikov 2000).

Uric acid is the end product of purine metabolism in humans and primates. Other mammals further oxidize uric acid to allantoin in a reaction catalyzed by the enzyme uricase. Traditionally, it has been thought that uric acid is just a waste product without physiological significance, but uric acid is actively reabsorbed in the distal tubule in kidney and possesses antioxidant properties (Becker 1993; Davies et al. 1986). According to one hypothesis the decrease in uricase activity and the further increase of plasma levels of uric acid in one of Man's predecessors might have resulted in a quantum jump in the intellectual capability and thus triggered the emergence of Man (Orowan 1955). The loss of uricase activity proceeded approximately at the same time as the loss of ability to synthesize ascorbic acid in humans. It is possible that uric acid partly replaced ascorbic acid in human metabolism and exogenous intake of vitamin C was sufficient for coverage of its need (Proctor 1970).

At physiological pH almost all uric acid is ionized to urate. This ubiquitous compound is capable especially of reacting with hydroxyl radicals and hypochlorous acid, being itself converted to innocuous products, e.g. allantoin, allantoate, glyoxylate, urea, and oxalate (Becker 1993). Urate also stabilizes ascorbate in biological fluids due to an inhibition of iron-catalyzed oxidation of ascorbate (Davies et al. 1986; Sevanian et al. 1991). On the other hand, strong oxidants (e.g. OH•) oxidize urate to a free radical, which is resonance-stabilized and does not appear to react with O_2 to form peroxyl radical. Ascorbate is expected to reduce the urate radical, because the reduction potential of the urate/urate radical system (E^0 = 0.59 V) is considerably higher than that of ascorbate (E^0 = -0.28 V). This presumption has been confirmed experimentally (Simic and Jovanovic 1989).

3.2.4. Plant polyphenols

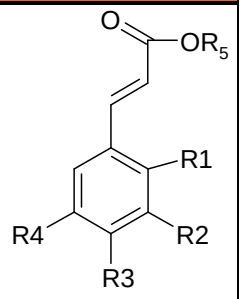
Plant polyphenols represent a heterogeneous group of natural compounds with one or more hydroxyl groups attached to the benzene ring. These substances are products of secondary metabolism in plants which are not directly involved in processes of primary metabolism (e.g. growth, development). They are synthesized primarily from

the products of the shikimate pathway and possess several important physiological roles in plants, such as defense against herbivores and pathogens, pigmentation, and attraction of pollinating insects. Flavonoids, hydroxycinnamic acids, phenolic acids, lignans, and tannins belong to the group of plant polyphenols.

3.2.4.1. Hydroxycinnamic acids

The most widely distributed polyphenolic compounds in plant tissues are the hydroxycinnamic acids (HCAs), which are synthesized via shikimate pathway from L-phenylalanine and L-tyrosine. HCAs are present in many diets in higher concentrations than the flavonoids. Some of the most common natural hydroxycinnamic acids are *p*-coumaric, ferulic, sinapic, and caffeic acid. These can be found in a free form but more often in various conjugated forms resulting from enzymatic hydroxylation, *O*-glycosylation, *O*-methylation or esterification (Dewick 2002; Rice-Evans et al. 1997). Only carboxyl groups of HCAs are included in the formation of those esters (Chen and Ho 1997). The representatives of hydroxycinnamic acids are presented in Table 3.

Table 3. Chemical structures of the most common hydroxycinnamic acids.

Compound	Substituents					Chemical Formula
	R_1	R_2	R_3	R_4	R_5	
***o*-Coumaric acid**	OH	H	H	H	H	
***m*-Coumaric acid**	H	OH	H	H	H	
***p*-Coumaric acid**	H	H	OH	H	H	
Caffeic acid	H	OH	OH	H	H	
Ferulic acid	H	OCH_3	OH	H	H	
Isoferulic acid	H	OH	OCH_3	H	H	
Sinapic acid	H	OCH_3	OH	OCH_3	H	
Chlorogenic acid	H	OH	OH	H	QA[1]	

[1]QA = quinic acid (=1,3,4,5-Tetrahydroxycyclohexanecarboxylic acid)

Caffeic acid is frequently the most abundant phenylpropanoid in food and it is the major representative of hydroxycinnamic acids in *Citrus* fruit (Rice-Evans et al. 1997). It occurs mainly as an ester with quinic acid, called chlorogenic acid. Several caffeic acids esters were isolated from propolis (Sud'ina et al. 1993). Coffee beans are one the richest dietary sources of chlorogenic acid (up to 350 mg/cup of coffee) and frequent coffee-drinkers might consume more hydroxycinnamic acids than flavonoids (Clifford 1999). Ferulic acid is the major phenolic acid in wheat and widely occurs also in other grains and vegetables (Graf 1992). Sinapic, ferulic and *p*-coumaric acid have been reported in maple products (Clifford 2000).

Hydroxycinnamic acids have been reported to possess antimicrobial, antiallergic and anti-inflammatory activity, as well as antimutagenic properties (Pannala et al. 1998). Phenethyl esters of caffeic and ferulic acids isolated from propolis have showed also immunomodulatory effect (Natarajan et al. 1996). Caffeic, *p*-coumaric, and *m*-coumaric acids have shown hepatoprotective properties as they reduce liver damage caused by CCl_4 intoxication in rats (Perez-Alvarez et al. 2001).

3.2.4.1.1. Antioxidant and anti-radical activity of HCAs

HCAs possess also antioxidant and anti-radical properties. They are able to interact with singlet oxygen, peroxynitrite, hypochlorite, superoxide anion radical, and hydrogen peroxide, as well as with some artificial stable radicals used as models for lipid oxidation (e.g. DPPH•)[I].

Foley et al. (1999) have tested the ability of HCAs to quench singlet oxygen ($^1O_2$) that seems to play a role in tumor promotion and carcinogenesis. Their results suggest that the most efficient quencher of $^1O_2$ is dihydroxyl substituted caffeic acid and the least efficient quenchers are monohydroxyl substituted coumaric acids. The position of the hydroxyl group plays little role in quenching efficiency and quenching rate constants (k_q) are very similar for *o*-, *m*-, and *p*-coumaric acid. Methoxylated HCAs (ferulic and sinapic acid) appear to be better quenchers than coumaric acids. It seems that the number of methoxyl groups plays an important role. Values of kq obtained for *p*-coumaric, ferulic, sinapic and caffeic acid in D_2O are $6x10^6$ M^{-1} s^{-1}, $20x10^6$ M^{-1} s^{-1}, $30x10^6$ M^{-1} s^{-1} and $40x10^6$ M^{-1} s^{-1}, respectively.

Superoxide anion radical scavenging capacity of caffeic and ferulic acid has been tested by Cano et al. (2002). Superoxide anions were generated non-enzymatically in a phenazine methosulfate-NADH system and assayed by reduction of nitro-blue tetrazolium (NBT). Caffeic and ferulic acids have shown no effect on superoxide anion generation at the used concentrations (5-10 μM). Toda et al. (1991) have described O_2^{-}• scavenging activity of ferulic acid in a concentration range 1-10 mM. Superoxide anion was generated in a xanthine/xanthine oxidase system and assayed by reduction of NBT.

Peroxynitrite is a cytotoxic species and hence the ability of HCAs to inhibit peroxynitrite dependent tyrosine nitration have been tested (Pannala et al. 1998). The inhibitory potency found followed the order: caffeic acid ≈ chlorogenic acid ≈ ferulic

[I] 2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl radical

acid > *p*-coumaric acid > *o*-coumaric acid > *m*-coumaric acid. These findings suggest that there are two possible mechanisms of inhibition of peroxynitrite-induced nitration of tyrosine. Monohydroxylated HCAs (ferulic and coumaric acids) are preferentially nitrated and therefore protect tyrosine from undergoing nitration. HCAs containing catechol moiety (caffeic and chlorogenic acid) probably donate electron to peroxynitrite and form nitrite and the corresponding quinone. Sinapic acid was also found to be an effective peroxynitrate scavenger (Zou et al. 2002).

The efficiency of HCAs and their derivatives as radical scavengers was evaluated by their reactivity toward a stable free radical DPPH•, which is a generally used mechanism for lipid peroxidation inhibition by antioxidants. Several research groups were using this method for evaluation of structure-activity relationship (Chen and Ho 1997; Silva et al. 2000; Son and Lewis 2002). Dihydroxy HCAs (caffeic and chlorogenic acid) have higher scavenging activity than *p*-hydroxymethoxy- or -dimethoxy HCAs (ferulic and sinapic acid) and these have higher activity than monohydroxylated HCAs (coumaric acids). Esterification of caffeic acid caused a decrease in the antiradical activity.

Antioxidant activity of HCAs on *in vitro* oxidation of LDL was studied using different models. Natella (1999) and Nardini (1995) used 2 oxidative systems: the thermal decomposition of AAPH[II] producing peroxyl radicals and the copper-catalyzed oxidation of LDL. Caffeic acid seems to be the most effective antioxidant of all tested HCAs. In fact, only caffeic acid completely protected LDL against oxidation at 5 μM concentration. Caffeic acid is the only hydroxycinnamic acid able to chelate Cu^{2+} ions used in the latter mentioned system. Castelluccio (1995) investigated the antioxidant activity of HCAs in the model of LDL peroxidation mediated by methmyoglobin. The results obtained in this study are similar to those in previously mentioned studies.

Briefly, the antioxidant and antiradical activity of HCAs is dependent on the number and position of hydroxyl groups. Abstraction of a hydrogen atom from HCAs results in the formation of a resonance-stabilized phenoxy radical (example is given in Fig. 1). Caffeic acid is capable of donating both hydrogen atoms resulting in the formation of *o*-quinone (Graf 1992). Antioxidant potential of other HCAs is lower than that of caffeic acid.

[II] 2,2'-azobis(2-amidinopropane) dihydrochloride

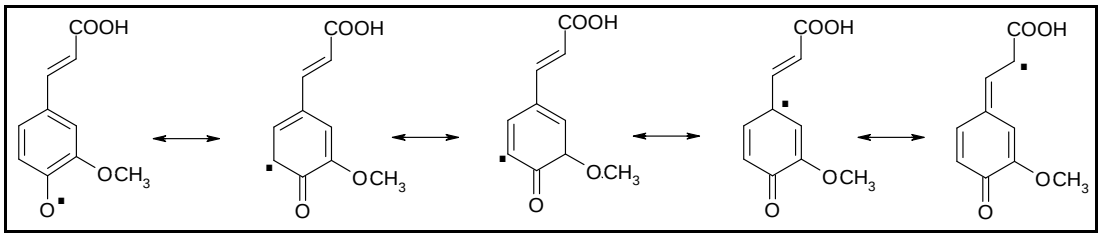


Fig. 1: Resonance stabilization of ferulic acid radical (Graf 1992).

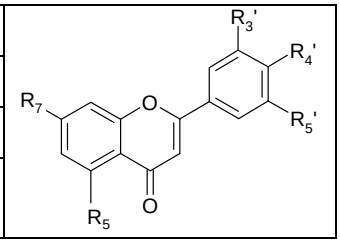
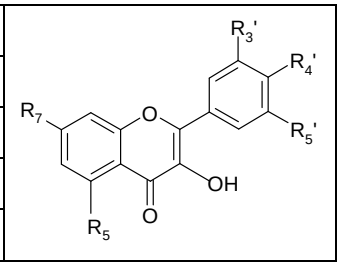
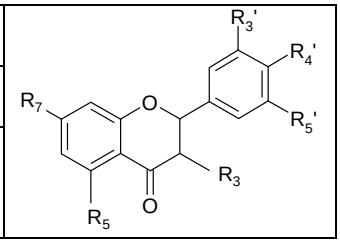
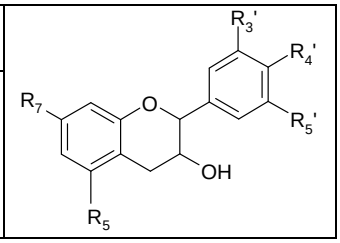
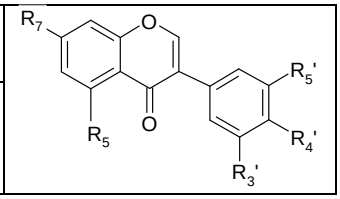
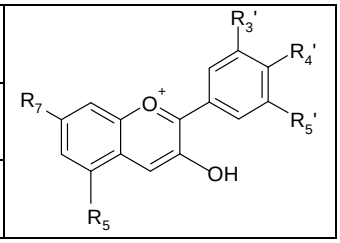
3.2.4.2. Flavonoids

Flavonoids, a group of benzo-γ-pyron derivatives, are natural polyphenolic compounds ubiquitously found in fruits and vegetables. They are synthesized from a cinnamoyl-CoA starter unit, with chain extension using 3 molecules of malonyl-CoA (Dewick 2002). Values of flavonoid daily intake reported in scientific literature varies from 1 to 1000 mg/day (Aherne et al. 2002). In plants, flavonoids generally occur as glycosylated and sulfated derivatives (Dewick 2002; Middleton et al. 2000). The prevalent sugar moieties are glucose, rhamnose, xylose, galactose, arabinose, and fructose (Kahkonen and Heinonen 2003; Rice-Evans et al. 1996). Over 4000 unique flavonoids have been identified in plant sources and quercetin, catechin, kaempferol, and rutin are probably the best known representatives.

Despite this huge diversity, all flavonoids can be recognized by their structural properties. The structure of flavonoids is based on the flavonoid nucleus, which consists of 3 phenolic rings (C_6-C_3-C_6) referred to as ring A, B, and C. The benzene ring A is condensed with a six-member heterocyclic ring (C), which in the 2-position carries a phenyl benzene ring (B) as a substituent. The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings (Table 4). Structural variations within the rings subdivide the flavonoids into 6 main subclasses (Aherne et al. 2002; Pietta 2000):

- Flavonols (e.g. quercetin and kaempferol), with the 3-hydroxy, 4-oxo group and 2,3-double bond in the C ring
- Flavones (e.g. luteolin, apigenin, and rutin), lacking the 3-hydroxy group
- Flavanols (e.g. catechin), lacking the 2,3-double bond and 4-oxo group
- Flavanones (e.g. naringenin), lacking the 2,3-double bond and the 3-hydroxyl group
- Isoflavones (e.g. genistein), with the B ring located in the position 3 of the C ring
- Anthocyanidins (e.g. cyanidin, delphinidin), positively charged oxonium ion in the C ring with lacking 4-oxo group

Table 4. Subclasses of naturally occurring flavonoids and a few examples of each class

Compound	Substituents						Chemical formula
	R_3	R_5	R_7	$R_{3'}$	$R_{4'}$	$R_{5'}$	
(A) FLAVONES							
Apigenin	H	OH	OH	H	OH	H	
Baicalein	H	OH	OH	H	H	H	
Chrysin	H	OH	OH	H	H	H	
Luteolin	H	OH	OH	OH	OH	H	
(B) FLAVONOLS							
Galangin	OH	OH	OH	H	H	H	
Kaempferol	OH	OH	OH	H	OH	H	
Myricetin	OH	OH	OH	OH	OH	OH	
Quercetin	OH	OH	OH	OH	OH	H	
Rutin	ORu[1]	OH	OH	OH	OH	H	
(C) FLAVANONES							
Hesperetin	H	OH	OH	OH	OCH_3	H	
Naringenin	H	OH	OH	H	OH	H	
Taxifolin	OH	OH	OH	OH	OH	OH	
(D) FLAVANOLS							
Catechin	OH	OH	OH	OH	OH	H	
Epigallocatechin	OH	OH	OH	OH	OH	H	
(E) ISOFLAVONES							
Daidzein	H	H	OH	H	OH	H	
Genistein	H	OH	OH	H	OH	H	
(F) ANTHOCYANIDINS							
Pelargonidin	OH	OH	OH	H	OH	H	
Cyanidin	OH	OH	OH	OH	OH	H	
Delfinidin	OH	OH	OH	OH	OH	OH	

[1]Ru = Rutinose (=Glc-Rha)

Diets rich in fruits and vegetables are protective against cardiovascular diseases, certain forms of cancer, and perhaps against other diseases. The dietary anomaly called French paradox was first observed in French population and found later also in other Mediterranean populations. Epidemiological studies revealed that flavonoid-rich diet is correlated with the increased longevity and decreased incidence of cardiovascular diseases seen in these populations (Burr 1995; Formica and Regelson 1995; Renaud and de Lorgeril 1992). In addition to their antioxidant properties, flavonoids have been reported to exhibit a multiple other biological effects, e.g. antiviral (Weber et al. 2003), antibacterial (Alvesalo et al. 2006), anti-inflammatory (Widlansky et al. 2005), and vasodilatory (Calderone et al. 2004). Moreover, they are able to inhibit lipid peroxidation and platelet aggregation and improve increased capillary permeability and fragility (Cirico and Omaye 2006; Hubbard et al. 2004; Valensi et al. 1996).

3.2.4.2.1. Antioxidant and anti-radical properties of flavonoids

Antioxidant and anti-radical properties of flavonoids have been extensively studied both *in vitro* and *in vivo*. Flavonoids exert their antioxidant activity by various mechanisms in aqueous as well as in lipophilic phase. Their antioxidant effect in aqueous phase is given by RONS scavenging activity. The specific mode of inhibition of oxidation in lipophilic phase by individual flavonoids is not clear but it may be by: scavenging of lipid alkoxyl and peroxyl radical, by chelating transition metal ions, and by regenerating α-tocopherol. Furthermore, flavonoids can inhibit catalytic activity of several enzymes involved in RONS generation (Korkina and Afanas'ev 1997; Pietta 2000; Rice-Evans et al. 1996).

Flavonoids are able to scavenge hydroxyl radical (Cao et al. 1997; Sichel et al. 1991), superoxide radical (Jovanovic et al. 1994; Sichel et al. 1991), peroxynitrite (Heijnen et al. 2001a; Heijnen et al. 2001b), hypochlorate (Firuzi et al. 2004), and singlet oxygen (Tournaire et al. 1993) in aqueous phase. Flavonoids with maximal antioxidant effect in aqueous phase have the following structural features in their molecules: 3',4'-*o*-dihydroxy structure in the B ring (presence of 3 hydroxyl groups in the B ring further increases antioxidant activity); 2,3-double bond in conjugation with 4-oxo function in the C ring; 3- and 5-hydroxyl groups with 4-oxo function in A and C rings. According to previously stated criteria, flavonols quercetin and myricetin are the most effective radical scavengers in the aqueous phase, which has been experimentally confirmed (Rice-Evans et al. 1996). Several different methods are used for antioxidant

activity determination *in vitro*, e.g. TEAC assay (Trolox equivalent antioxidant capacity), ORAC assay (Oxygen radical absorbance capacity), superoxide scavenging (SOS) in xanthine/xanthine oxidase system, hypochlorite scavenging in hypochlorite/hypochlorous acid system, and peroxynitrite scavenging using oxidation of dihydrorhodamine-123.

TEAC assay, a widely used method for determination of structure-activity relationships (SAR) of flavonoids against RONS in aqueous phase, reflects the ability of hydrogen-donating antioxidants to scavenge the stable $ABTS^{III}$ radical cation. The activity of the tested antioxidant is compared with antioxidant activity of Trolox, synthetic water-soluble analog of α-tocopherol (Pannala et al. 2001; Rice-Evans et al. 1996). However, Arts et al. (2003) have tested chrysin and galangin among others and their obtained TEAC values were comparable though galangin showed much better antioxidant properties in other tests. This discrepancy is probably caused by the ability of some reaction products to further react with ABST•. This observation hampers the use of the TEAC assay for constructing SAR.

The total antioxidant activity of flavonoids in lipid phase is probably a combination of their abilities to chelate transition metal ions; to scavenge lipid alkoxyl and peroxyl radicals; and to regenerate α-tocopherol through reduction of α-tocopheryl radical. Mira et al. (2002) have described three metal-complexing sites within a flavonoid molecule containing hydroxyl groups at 3, 5, 3', and 4' position. These sites are located between the 5-hydroxyl and the 4-oxo group, the 3-hydroxyl and the 4-oxo group, and between the *o*-hydroxyl groups in the B ring. Some flavonoids are able not only to chelate metal ions but also to reduce them. Flavonoids capable of reducing transition metal ions (myricetin, quercetin) can exert pro-oxidant activity (Cao et al. 1997; Mira et al. 2002). The peroxyl radical scavenging ability of flavonoids was assessed by ORAC assay with AAPH as ROO• generator. The $ORAC_{ROO•}$ activity is proportional to the number of hydroxyl groups in the molecule of the flavonoid (Cao et al. 1997). Ioku et al. (1995) tested the peroxyl radical scavenging activities of quercetin and its monoglucosides by inhibiting methyl linoleate hydroperoxidation induced by the azo compounds AAPH or $AMVN^{IV}$. Flavonoids also possess strong protective effect against LDL-oxidation (Aviram et al. 2000; Osada et al. 2001).

[III] 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)
[IV] 2,2'-azobis(2,4-dimethylvaleronitrile)

Several authors used rather the reduction potentials (E_7) as a measure of their antioxidant capacities (Firuzi et al. 2005; Jovanovic et al. 1994). The reduction potentials of flavonoids (0.54 – 0.7 V) are lower than those of peroxyl radical (1.05 V) and superoxide/hydroperoxyl radicals (0.94 V), which means that they may efficiently inactivate these radicals. Furthermore, the E_7 value of tocopheryl radical is lower than that of flavonoids, which means that flavonoids are capable of regenerating α-tocopherol (Jovanovic et al. 1994).

3.2.4.3. Resveratrol

Resveratrol (*trans*-3,4',5-trihydroxystilbene), a phytoalexin produced by plants in response to damage, occurs particularly in grapevine (Vitis vinifera, *Vitaceae*). Resveratrol is found as *cis* and *trans* isomers, where only the *trans*-configuration is biologically active. Chemical structures of both isomers are showed in Fig. 2 (see below). It is also considered to be the main contributor to the total antioxidant power of red wine despite might not being the most abundant polyphenolic compound in wine (Burns et al. 2002). Resveratrol is often mentioned within the context of "French paradox" (Sun et al. 2002).

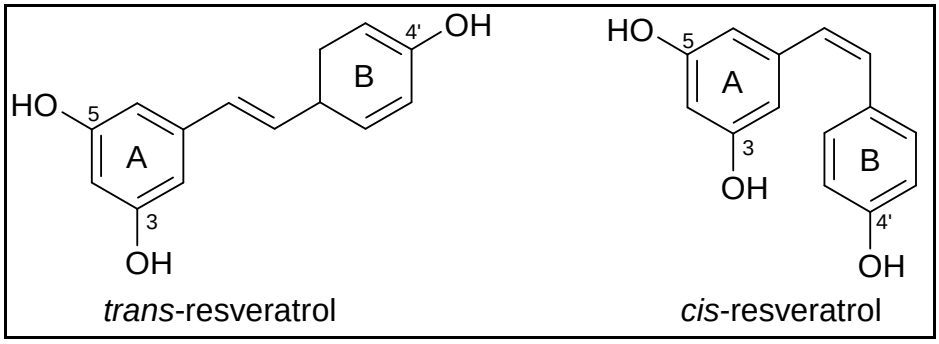


Fig. 2: Chemical structures of *cis* and *trans* isomers of resveratrol.

Resveratrol exhibits a wide range of biological effects, including antiplatelet, anti-inflammatory, anticancer, antiatherogenic, anti-mutagenic, antifungal and antioxidant properties (Baur and Sinclair 2006; Leonard et al. 2003). Resveratrol is a potent scavenger of free radicals which is able to inhibit lipid peroxidation *in vitro* as well as *in vivo*. Its ability to scavenge hydroxyl radical, superoxide anion radical (Leonard et al. 2003), peroxynitrite, and peroxyl radical has been described (Olas et al. 2006). The *para*-4'-OH group is crucial for the antioxidant activity of resveratrol and its importance is explained on the basis of its increased acidity compared to the other two hydroxyl groups (Caruso et al. 2004).

3.3. Non-enzymatic glycation

Non-enzymatic glycation, also called Maillard reaction, was first described by Louis Camille Maillard (1912). This non-enzymatic browning process had been first extensively studied by food chemists and later has become the center of attention of geological, agricultural, and medical sciences. In the medical field, the connection between glycation and diabetes mellitus has been proven many times. There is increasing evidence that Maillard reaction plays an important role in the onset and progression of some other diseases, such as atherosclerosis and Alzheimer's disease.

3.3.1. Formation and structure of AGEs

The early stage of the Maillard reaction is initiated by nonenzymatic condensation of a reducing sugar or a certain related compound (e.g. ascorbate) with a free amino group of a protein, a lipid or a nucleic acid. In the case of glucose, the reaction first leads to the formation of acid-labile Schiff base, which undergoes a rearrangement to a relatively stable Amadori product, e.g. fructosamine. Only a small portion of these Amadori-adducts experience further rearrangements leading to an irreversible formation of advanced glycation end-products (Monnier 1989). Suarez et al. (1989) have stated that the reaction with fructose proceeds in a similar way, but it is called Heyns rearrangement and two separate Heyns products are generated (Fig. 3). The formation of a Schiff base proceeds in the range of hours and it is fully reversible, while Amadori rearrangement takes days and is reversible only to a certain extent.

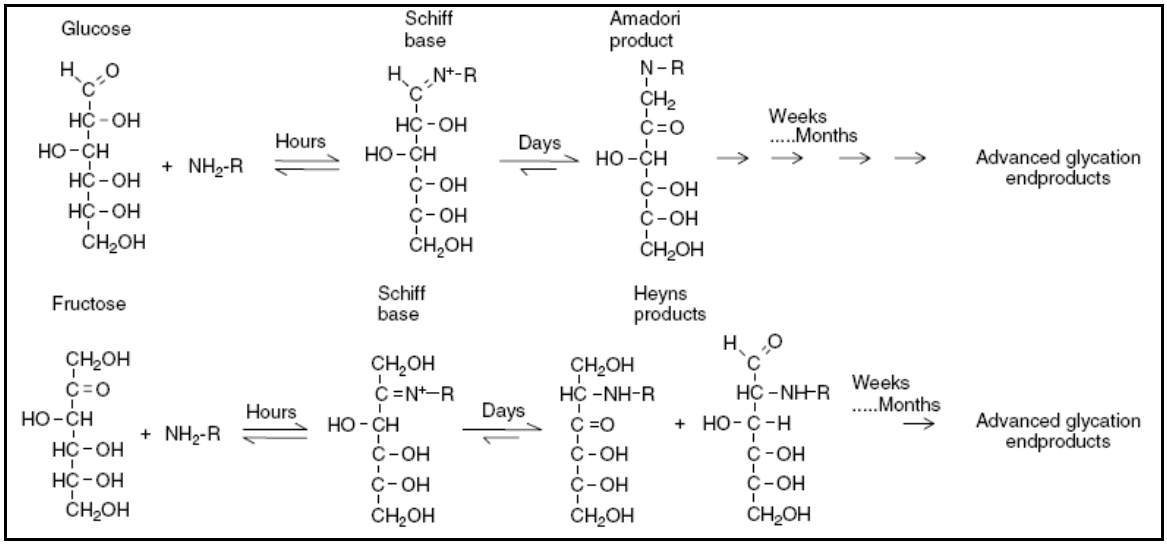


Fig. 3: The initial Maillard reaction with D-glucose and D-fructose. Scheme was taken over from the work of Schalkwijk et al. (2004).

In the intermediate stage, Amadori product subsequently degrades and various reactive intermediates are formed. These products are known as α-dicarbonyls or oxoaldehydes and are represented by products like methylglyoxal (MGO), 3-deoxyglucosone (3-DG), and glyoxal (GO). Also a Schiff base is a potential source of reactive α-dicarbonyls, because it can be fragmented to MGO and GO. These dicarbonyls possess higher reactivity towards proteins than the parent monosaccharide. They are capable of forming various cross-links as well as chromo/fluorophoric adducts called AGEs, upon reaction with proteins (Schalkwijk et al. 2004; Wolff et al. 1991). Both MGO and 3-DG form adducts with proteins and nucleic acids up to 10,000 times more readily than glucose (Beisswenger et al. 2003). The accumulation of dicarbonyl compounds is termed carbonyl stress (Miyata et al. 1999). The other process proceeding during the intermediate stage of glycation is metal catalyzed autoxidation of glucose, in which the carbonyl compounds (arabinose and glyoxal), H_2O_2 and free radicals are formed (Hunt et al. 1988; Wolff and Dean 1987). The generated free radicals initiate further oxidative steps. The glycation process accompanied by oxidation steps is called glycoxidation (Baynes 1991). Various pathways incorporated in the formation of AGEs are shown in Fig. 4.

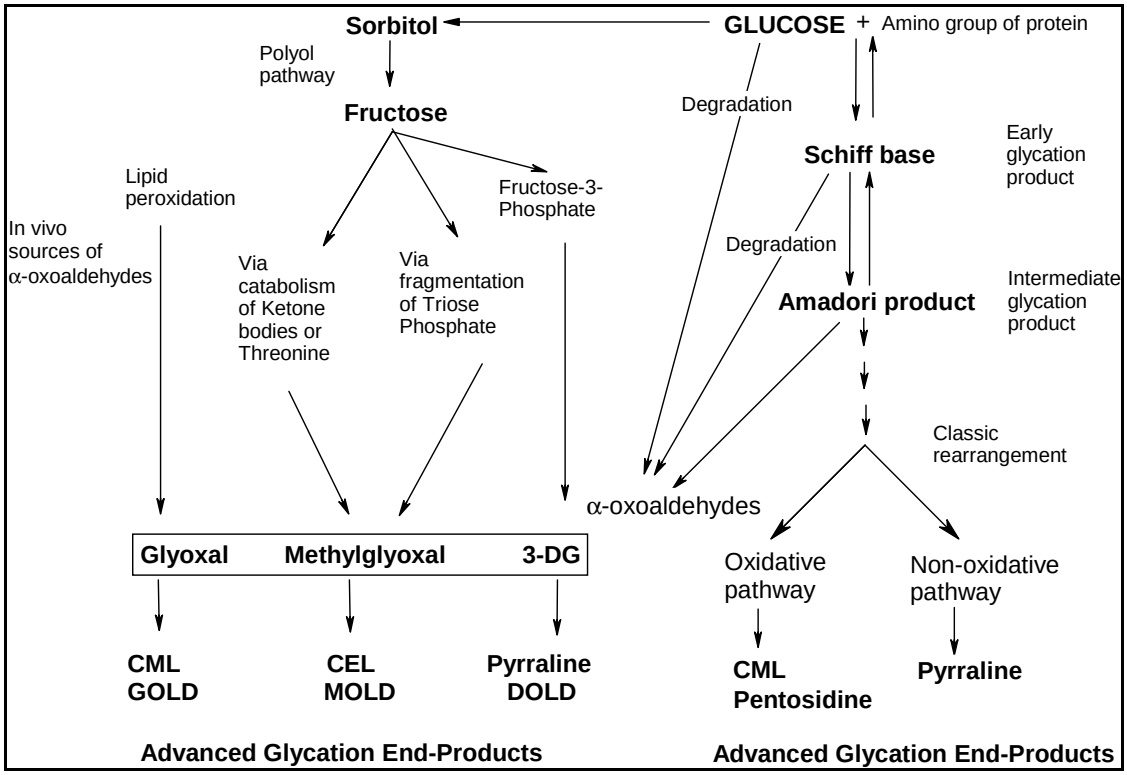


Fig. 4: Glucose and AGEs formation pathways including the polyol pathway and AGE formation by the α-oxoaldehydes (Singh et al. 2001).

The advanced glycation end-products are formed during the late stage of glycation over a period of weeks, thereby affecting predominantly long-lived proteins, such as collagen and lens crystallins. They represent a heterogeneous group of compounds rising from different precursors. The chemical structures of AGEs have not been fully described yet. These compounds are formed either by oxidative pathway (pentosidine and CML[V]) or by non-oxidative pathway (pyrraline, DOLD[VI], GOLD[VII], MOLD[VIII], and CEL[IX]) as can be seen in Fig. 4. Proteins modified by advanced glycation are characterized by a much higher molecular weight than the original protein, a yellow-brown pigmentation, a typical fluorescent spectra ($\lambda_{exc}/\lambda_{em}$: 370/440 nm), an ability to form various cross-links, and by their biological half-life, which is comparable to the half-lives of parent proteins (Lapolla et al. 2005; Singh et al. 2001).

Glucose is the least reactive of the common sugars and that is probably the reason for its evolutionary selection as the principal sugar *in vivo* (Bunn et al. 1978). Because of its low reactivity towards proteins, AGEs have been thought to form only at long-lived extracellular proteins, such as collagen, crystallines, and myelin. Recently also rapid intracellular AGE formation by various intracellular sugars (e.g. fructose, ribose, glyceraldehyde, dihydroxyacetone phosphate, glyceraldehyde-3-phosphate, glyoxal, methylglyoxal, and 3-deoxyglucosone) *in vivo* has been described. The rate of glycation is directly proportional to the percentage of sugar in the open-chain form (Bunn and Higgins 1981) and the rate for fructose (0.7% open-chain) is 7.5-fold faster than that of glucose (0.002% open-chain). More strikingly, the glycolytic intermediate glyceraldehyde-3-phosphate (100% open-chain) forms 200-fold more glycated proteins than do equimolar amounts of glucose (Ruderman et al. 1992).

Several groups of advanced glycation end-products found under physiological conditions can be created based on their fluorescent properties and their ability to form cross-links. The first group is represented by fluorescent AGE cross-links, which are thought to be responsible for a major share of the deleterious effects of AGEs in diabetes and aging. Fluorescence is a good qualitative indicator used to estimate AGEs formation. Pentosidine, crossline, and various vesperlysines are members of this group. However, also non-fluorescent AGE cross-links are found *in vivo*. Their isolation and

[V] N-ε-(carboxymethyl)lysine
[VI] deoxyglucosone-lysine dimer
[VII] glyoxal-lysine dimer
[VIII] methylglyoxal-lysine dimer
[IX] N-ε-(carboxyethyl)lysine

identification is more complicated than in the case of fluorescent AGE cross-links. It is thought that they account just for 1% of all cross-links rising under physiological conditions.Various imidazolium dilysine cross-links (GOLD, MOLD), arginine-lysine cross-links, and glucosepan belong to this group. Last but not least, a group of non-cross-linking protein bound AGE structures have been identified *in vivo*. These structures may exert deleterious effects as precursors of cross-links or as biological receptor ligands inducing a variety of adverse cellular and tissue changes. The well known members of this group are pyrraline, carboxyalkyllysines (CML, CEL), imidazolones, and argpyrimidine (Ulrich and Cerami 2001). Classification and examples of each above mentioned group are shown in Fig. 5 below.

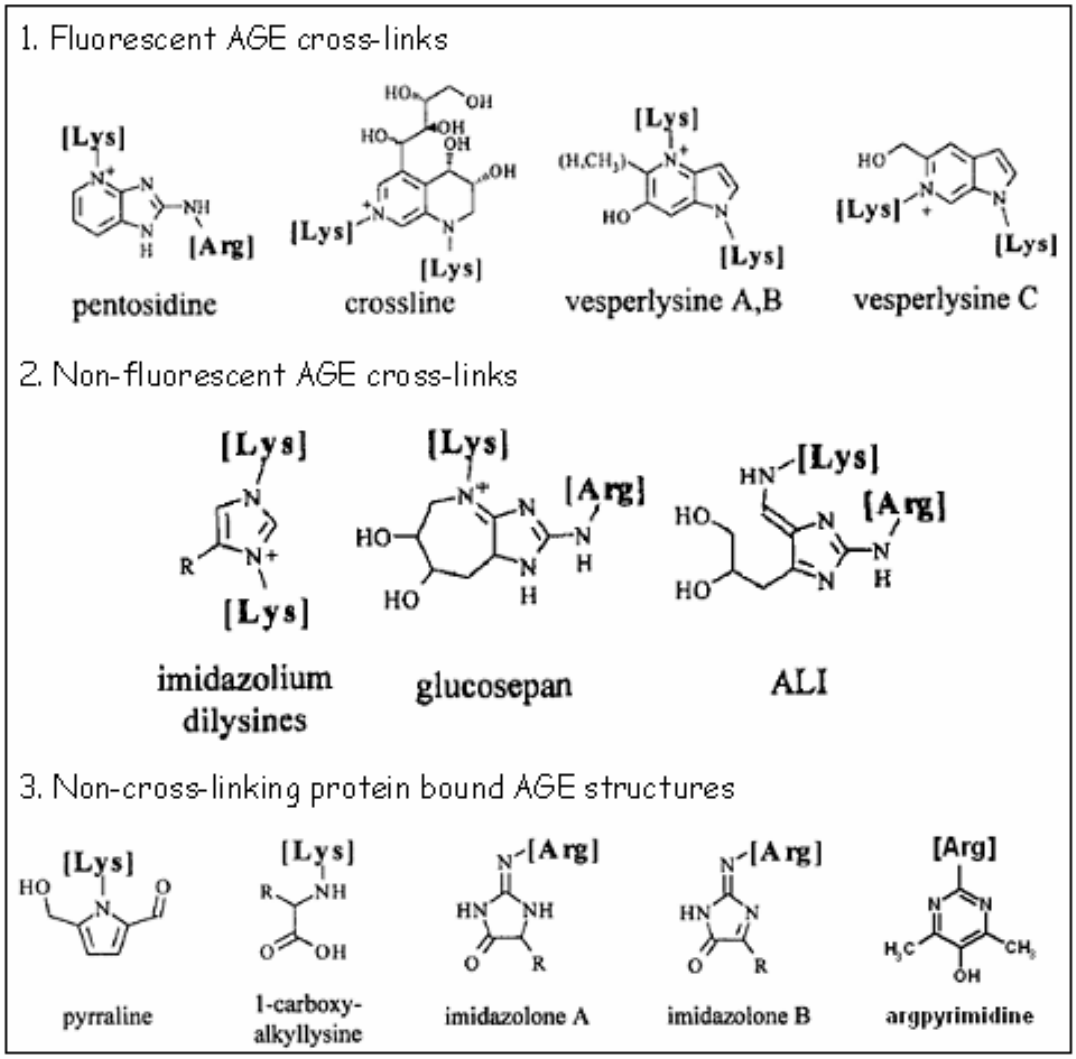


Fig. 5: Classification of AGEs formed under physiological conditions. [Lys] represents a desamino-lysine residue; [Arg] represents a desguanidino-arginine residue; R represents either hydrogen atom (GOLD) or methyl group (MOLD). Figure has been modified according to the work of Ulrich and Cerami (2001).

3.3.2. Receptors for AGE; degradation and elimination of AGEs

Recently, the existence of specific cellular receptors which bind AGE proteins has been proved. These receptors bind AGE proteins in a saturable manner. The first purified receptor for AGE was a scavenger receptor, which is expressed on the cell surface of T lymphocytes and macrophages. It degrades senescent molecules, e.g. AGE modified proteins. After digestion, small soluble AGE peptides are released and subsequently cleared by the kidney. Therefore, the effective elimination is dependent on the normal creatinine clearance (Vlassara 1997).

The second identified receptor was the receptor for AGE, the so-called RAGE, which belongs to the immunoglobulin superfamily. This receptor is expressed by various cell systems: smooth muscle cells, T lymphocytes, monocytes, macrophages, microglia, endothelial and mesangial cells. Binding of AGE to RAGE leads to oxidative stress and activation of transcription factor NF-κB, which further increases expression of endothelin-1, tissue factor, thrombomodulin, and adhesive molecule VCAM-1[X]. Binding of AGE to macrophages induces production of cytokines (IL-1 and TNF-α)[XI] and tissue growth factors, with consequent synthesis of type IV collagen, increased proliferation of vessel smooth muscle cells, and stimulation of macrophage chemotaxis (Lapolla et al. 2005; Singh et al. 2001).

The third protein with high affinity for AGE is galectin-3 (also known as AGE-R3 or Mac-2), which is expressed on macrophages. The binding of AGE promotes formation of high molecular weight complexes (Lapolla et al. 2005).

3.3.3. Possibilities of therapeutic intervention

The therapeutic intervention to the glycation process has followed three main approaches. A first approach is inhibition of AGE formation process by carbonyl-blocking agents (aminoguanidine) or by antioxidants. A second one is reducing AGE deposition by using cross-link breakers or by enhancing cellular uptake and degradation. The last approach follows inhibition of RAGE by neutralizing antibodies or suppression of post-receptor signaling using antioxidants.

[X] Vascular cellular adhesion molecule-1
[XI] Interleukin-1 and Tumor necrosis factor-α

3.3.3.1. Inhibition of AGE cross-links formation

Aminoguanidine, also known by its trade name Pimagedine (Alteon Inc.), is a low-molecular, highly nucleophilic hydrazine compound that rapidly reacts with α-dicarbonyl compounds such as MGO, GO, and 3-DG to prevent formation of AGE cross-links. The products of the scavenging reaction are substituted 3-amino-1,2,4-triazines. Aminoguanidine does not affect the formation of the Schiff base and Amadori products (Thornalley et al. 2000). Clinical trials of aminoguanidine in overt diabetic nephropathy (ACTION) were performed: ACTION I was conducted in patients with type 1 diabetes mellitus; ACTION II in patients with type 2 diabetes mellitus. Reported side effects of aminoguanidine in clinical therapy were gastrointestinal disturbance, abnormalities in liver function tests, flu-like symptoms, and a rare vasculitis (Bolton et al. 2004; Thornalley 2003).

Other nucleophilic compounds, which are designed to trap reactive carbonyl intermediates in AGE formation, are for example OPB-9195, diaminophenazine, tenilsetam, and pyridoxamine (Baynes and Thorpe 2000; De La Cruz et al. 2004). With regard to the presence of free radicals and oxidative steps in the course of glycoxidation, compounds with antioxidant effect such as α-lipoic acid, α-tocopherol, ascorbic acid, β-carotene were tested. Dipeptide carnosine, pyridoindole derivative stobadine, hypolipidemic drug probucol, and mucolytic drug N-acetylcysteine are just a few more examples of the compounds with described antioxidant properties, which were tested in order to estimate their potential protective effect in the process of glycation. Also some antioxidant enzymes such as SOD, CAT, and selenium-dependent glutathione peroxidase may protect proteins against impairment caused by non-enzymatic glycation (De La Cruz et al. 2004; Kyselova et al. 2004).

3.3.3.2. Cleavage of already formed AGE cross-links

Aminoguanidine and other compounds mentioned above can inhibit the formation of new AGE cross-links, but they are not able to cleave already formed cross-links. Vasan et al. (1996) reported the first of cross-link breakers, phenyl thiazolium bromide (PTB). This anti-AGE agent chemically breaks α-dicarbonyl compounds by cleaving the carbon-carbon bond between the carbonyls. Under physiological conditions, PTB is not stable and therefore its analogs were tested and alagebrium chloride (ALT-711), a highly potent cross-link breaker with higher stability, has been

discovered. This compound successfully completed preclinical studies and Phase II clinical study on healthy volunteers. Three Phase IIb trials initiated by Alteon Inc. are currently ongoing on population with systolic hypertension (Bakris et al. 2004).

3.3.3.3. Inhibition of RAGE

AGE- RAGE interactions have been implicated in the development of diabetic vascular complications, which cause various disabilities and shortened life expectancy, and reduced quality of life in patients with diabetes. These undesirable effects can be suppressed by the use of specific antibodies to RAGE, soluble RAGE or by suppression of post-receptor signaling using antioxidants (Hudson et al. 2003; Stuchbury and Münch 2005; Yan et al. 1994). Nakayama et al. (1991) characterized an anti-RAGE IgG as a specific antibody to RAGE. The secreted RAGE form, named soluble RAGE (sRAGE), acts as a decoy to trap ligands and prevent interaction with cell surface receptors (Bucciarelli et al. 2002). Soluble RAGE was shown to have important inhibitory effects in several cell culture and transgenic mouse models, in which it prevented or reversed full-length RAGE signaling. The administration of sRAGE has been shown to suppress accelerated diabetic atherosclerosis (Park et al. 1998).

3.3.4. Methods for measurement of glycation products

The three main methodological approaches used for quantification of AGEs are: immunological; spectroscopical and fluorometrical; chromatographical (HPLC) and mass spectrometrical.

Immunochemical methods use the antigenic properties of AGEs. Glycation products are measured by ELISA (enzyme linked immunosorbent assay) or RIA (radioimmunoassay) methods using polyclonal antibodies against AGEs, which are obtained *in vitro* from the glycation reaction of glucose with ribonuclease. These antibodies react with the AGE formed *in vivo*. However, AGEs are estimated totally and cross-reactivity with similar compounds cannot be excluded (Makita et al. 1992). Also individual AGEs can be assessed by ELISA technique using monoclonal antibodies. Monoclonal antibodies against CML, pyrraline, pentosidine, and 3-DG-imidazolone are commercially available. The RIA technique had been used for the measurement of the advanced glycation end-product FFI, which is now considered to be just an artifact of hydrolysis and subsequent neutralization (Horiuchi et al. 1988).

Due to the chemical characteristics of advanced glycation end-products, they can be measured by spectroscopic and fluorometric methods, exploiting their particular values of absorption (280 nm) and fluorescence ($\lambda_{exc}/\lambda_{em}$: 370/440 nm). However, these methods are non-specific and can give just indications about the general trend of glycation process. Tissue fluorescence (autofluorescence, Total AGEs) is assessed at the excitation wavelength 360-370 nm and emission wavelength 430-440 nm (Lapolla et al. 2005). Characteristics of two main AGE fluorophores are $\lambda_{exc}/\lambda_{em}$: 335/385 nm for pentosidine and $\lambda_{exc}/\lambda_{em}$: 320/382 nm for argpyrimidine (Sell and Monnier 1989; Shipanova et al. 1997). In addition to AGE-specific fluorescence, intrinsic protein fluorescence can be used to characterize AGE-modified proteins. A decrease in intrinsic protein fluorescence caused by tryptophan was reported by Schmitt et al. (2005). Colorimetric method, based on the ability of Amadori products to reduce the dye NBT and to produce purple formazan which absorbs at 525 nm, is applied to measure fructosamine in clinical practice (Lapolla et al. 1988).

Chromatographic techniques and mass spectrometry are very popular and accurate methods used for separation, quantification, and identification of compounds as well as biological material, e.g. products of metabolism. HPLC methods of separation and quantification have been developed for virtually all advanced glycation end-products. There are several HPLC and GC/MS methods also for measuring GO and MGO (Lapolla et al. 2003) as well as 3-DG (Niwa 1999). HPLC methods with fluorescence detection are used for assessment of pentosidine and argpyrimidine (Sell and Monnier 1989; Shipanova et al. 1997).

3.3.5. *In vitro* models of glycation/glycoxidation

Non-enzymatic glycation is an extensively studied process that plays an important role in the course of some disorders (e.g. diabetes mellitus, atherosclerosis, and Alzheimer's disease). Therefore, an immense number of various *in vitro* and *in vivo* models have been developed with a view to investigate and better understand its complicated mechanism as well as its contribution to the development and progress of several civilization diseases. Structural, plasma, and cellular proteins including various enzymes as well as proteins of chromatin have been used as model proteins on which glycation proceeds. These proteins can be glycated by a variety of sugars, dicarbonyl compounds or sugar-related compounds (e.g. ascorbate) in the presence or absence of

transition metal ions that promote the oxidative steps of the glycoxidation process. Protective effect of the different antioxidants and antiglycating agents against glycation can be investigated in these *in vitro* models. Several examples of already described models of *in vitro* glycation of proteins are given below in Table 5.

Table 5. Developed models of protein glycation *in vitro*

Glycated protein	Glycating agent	References
Alanine aminotransferase	D-Glucose	(Beranek et al. 2001)
	D-Fructose	(Beranek et al. 2001; Beranek et al. 2002; Beranek et al. 2006)
	D-Ribose	(Beranek et al. 2001)
	D,L-Glyceraldehyde	(Beranek et al. 2001)
Alkaline phosphatase	D-Glucose	(Koyama et al. 1998)
Antithrombin III	D-Glucose	(Sakurai et al. 1988)
	Methylglyoxal	(Gugliucci and Menini 2002)
Aspartate aminoransferase	D-Glucose	(Drsata et al. 2002; Okada and Ayabe 1995)
	D-Fructose	(Beranek et al. 2002; Beranek et al. 2006; Drsata et al. 2002)
	D-Ribose	(Drsata et al. 2002)
	Ribose 5-phosphate	(Seidler and Seibel 2000)
	D,L-glyceraldehyde	(Drsata et al. 2002; Seidler and Seibel 2000; Seidler and Yeargans 2002)
	Glyceraldehyde 3-Phosphate	(Fitzgerald et al. 2000; Kopelovich et al. 1970; Swearengin et al. 1999)
	Methylglyoxal	(Seidler and Yeargans 2002)
Beta-lactoglobulin	D-Glucose	(Liggins and Furth 1997)
	D-Fructose	(Chevalier et al. 2002; Liggins and Furth 1997)
	D-Galactose	(Fenaille et al. 2004; Chevalier et al. 2002)
	D-Ribose	(Chevalier et al. 2002)
	Arabinose	(Chevalier et al. 2002)
	Lactose	(Fenaille et al. 2004; Chevalier et al. 2002; Morgan et al. 1999)
	Rhamnose	(Chevalier et al. 2002)

Table continues in the following pages 38 and 39.

Table 5. Continue

Glycated protein	Glycating agent	References
Bovine serum albumin	D-Glucose	(Kanska and Boratynski 2002; Liggins and Furth 1997; Rahbar et al. 2000; Stefek et al. 1999; Watala et al. 1992)
	D-Fructose	(Kanska and Boratynski 2002; Liggins and Furth 1997; Syrovy 1994; Yeboah et al. 1999)
	D- Galactose	(Syrovy 1994)
	D-Ribose	(Booth et al. 1997; Syrovy 1994; Verbeke et al. 2000)
	Arabinose	(Verbeke et al. 2000)
	D,L-Glyceraldehyde	(Syrovy 1994)
	Glyoxal	(Verbeke et al. 2000; Zeng et al. 2006)
	Methylglyoxal	(Zeng et al. 2006)
Catalase	D-Glucose	(Yan and Harding 1997)
	Glucose 6-phosphate	(Yan and Harding 1997)
	D-Fructose	(Yan and Harding 1997; Zhao et al. 2000)
	D-Ribose	(Yan and Harding 1997)
Collagen	D-Glucose	(Cervantes-Laurean et al. 2006; Ferreira et al. 2003; Raabe et al. 1996; Sajithlal et al. 1998; Watala et al. 1992)
	D-Ribose	(Paul et al. 1998; Raabe et al. 1996)
Crystallins	D-Glucose	(Liang and Rossi 1990; Swamy-Mruthinti and Carter 1999; Zhao et al. 1996)
	Glucose 6-phosphate	(Liang and Rossi 1990)
	D-Fructose	(Zhao et al. 1996; Zhao et al. 2000)
	Fructose 6-phosphate	(Yan and Harding 2006)
	D- Galactose	(Blakytny et al. 1997; Facchiano et al. 1996)
	D-Ribose	(Chellan and Nagaraj 2001; Liang and Rossi 1990; Yan and Harding 2006)
	Methylglyoxal	(Seidler et al. 2004)
Cu,Zn-superoxide dismutase	D-Glucose	(Jabeen and Saleemuddin 2006; Ookawara et al. 1992; Yan and Harding 1997)
	Glucose 6-phosphate	(Yan and Harding 1997)
	D-Fructose	(Jabeen and Saleemuddin 2006; Ookawara et al. 1992; Yan and Harding 1997; Zhao et al. 2000)
	D-Ribose	(Jabeen and Saleemuddin 2006; Yan and Harding 1997)
Elastin	D-Glucose	(Winlove et al. 1996)
	D-Ribose	(Winlove et al. 1996)

Table 5. Continue

Glycated protein	Glycating agent	References
Ferricytochrome c	D-Glucose	(Gillery et al. 1988)
	D-fructose	(Gillery et al. 1988)
Glucokinase	D,L-Glyceraldehyde	(Murata et al. 1993)
Glucose-6-phosphate dehydrogenase	D-Fructose	(Zhao et al. 1998)
Glutathione reductase	D-Glucose	(Blakytny and Harding 1992; Morgan et al. 2002)
	Glucose 6-phosphate	(Blakytny and Harding 1992)
	D-Fructose	(Blakytny and Harding 1992)
	Glyoxal	(Morgan et al. 2002)
	Methylglyoxal	(Morgan et al. 2002)
Glyceraldehyde-3-phosphate dehydrogenase	D-Glucose	(Morgan et al. 2002)
	D-Fructose	(Zhao et al. 2000)
	D,L-Glyceraldehyde	(Seidler and Yeargans 2002)
	Glyoxal	(Morgan et al. 2002)
	Methylglyoxal	(Lee et al. 2005; Morgan et al. 2002; Seidler and Yeargans 2002)
Hemoglobin	D-Glucose	(Jain and Palmer 1996; Rahbar et al. 2000; Watala et al. 1992)
Histones	D-Glucose	(Gugliucci 1994; Liebich et al. 1993; Talasz et al. 2002)
	Glucose 6-phosphate	(Gugliucci 1994)
	D-Fructose	(Gugliucci 1994; Talasz et al. 2002)
	D-Ribose	(Gugliucci 1994; Talasz et al. 2002)
	Fucose	(Gugliucci 1994)
Human serum albumin	D-Glucose	(Coussons et al. 1997; Mendez et al. 2005)
Lactate dehydrogenase	D-Glucose	(Morgan et al. 2002)
	Glyoxal	(Morgan et al. 2002)
	Methylglyoxal	(Morgan et al. 2002)
Lysozyme	D-Glucose	(Liggins and Furth 1997)
	D-Fructose	(Liggins and Furth 1997)
Methemoglobin	D-Ribose	(Booth et al. 1997; Cussimanio et al. 2003)
Metmyoglobin	D-Glucose	(Cussimanio et al. 2003; Roy et al. 2004)
Ovalbumin	D-Glucose	(Sun et al. 2004; Zeng et al. 2006)
	D-Fructose	(Sun et al. 2004)
	Glyoxal	(Zeng et al. 2006)
	Methylglyoxal	(Zeng et al. 2006)
Plasminogen	Methylglyoxal	(Gugliucci and Menini 2002)
Ribonuclease A	D-Glucose	(Booth et al. 1997; Khalifah et al. 1996; Watkins et al. 1985)
	D-Ribose	(Booth et al. 1997; Khalifah et al. 1996)

4. AIM OF THE WORK

The following aims were set for the project:

1) The first aim of the work was to establish a suitable *in vitro* model of protein glycation:
    i) Comparing several commercially available AST preparations and finding the best source for future studies;
    ii) Finding of a suitable glycating agent and its concentration, which will be used in further experiments (Frc; Frc + Cu^{2+}or Fe^{3+}; MGO).

2) The second aim was extension of a palette of methods for the evaluation of protein glycation and oxidative stress:
    i) Introducing fluorescence measurement for monitoring the generation of AGEs;
    ii) Learning several *in vitro* methods for evaluation of oxidative stress, which could also be used for the measurement of the course of glycation.

3) The third aim of the work was to evaluate the influence of potential antioxidants on the catalytic activity of AST and glycation of AST by a glycating agent:
    i) Evaluating direct protein-antioxidant interactions;
    ii) Evaluating antiglycation effects of antioxidants and determination of potential structure-activity relationships.

5. EXPERIMENTAL PART:

RESULTS AND DISCUSSION

5.1. Determination of quality of AST preparations using spectroscopic methods

Držata, J., Boušová, I., Maloň, P. (2005) Determination of quality of pyridoxal-5´-phosphate enzyme preparations by spectroscopic methods. J Pharm Biomed Anal. 37(5): 1173-1177.

Aspartate aminotransferase (AST, EC 2.6.1.1) belongs to a group of pyridoxal-5'-phosphate-dependent enzymes that catalyses reversible transamination. The active sites of these enzymes contain coenzyme pyridoxal-5'-phosphate (PLP), which is involved in enzymatic catalysis. Porcine cytosolic AST (cAST) is a homodimer with molecular mass of about 93,150 Da. Both subunits are composed of 412 amino acid residues (BRENDA 2005) and each of them consists of one small and one large domain. PLP coenzyme forms a Schiff base with ε-amino group of Lys-258 located in an active site of the larger domain (Kirsch et al. 1984). Crystal structure of cAST is shown in Figure 6. Coenzyme PLP bound in the active site of enzyme provides the AST molecule a characteristic absorption and circular dichroism spectra. Absorption spectra of free PLP differ from that of bound coenzyme. Free PLP does not have circular dichroism spectra, because it is not an optically active compound.

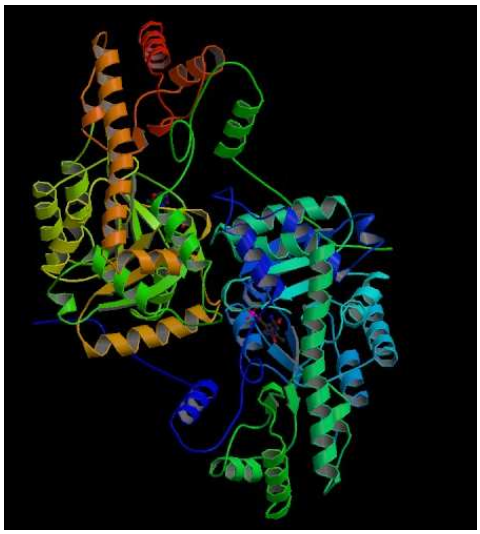


Fig. 6: Crystal structure of cAST from pig heart with coenzyme pyridoxal-5'-phosphate located in the active sites of enzyme (Rhee et al. 1997).

AST is available in relatively pure and stable form from several commercial sources that appeared to be more or less suitable for previous studies of our research group (Drsata et al. 2002). The objective of the presented study was to use fluorescent and spectral properties of commercially available AST preparations for evaluation of their quality and selection of the most suitable source of the enzyme for future studies of protein glycation and the influence of antioxidants on this process.

Incubation mixtures containing 1 mg/ml of AST were prepared from the enzyme preparations obtained from the companies Serva, Roche and Sigma-Aldrich. Two types

of incubation mixtures were prepared: AST in buffer only and AST in buffer with 1 mM L-aspartate. Some samples were dialyzed for the purposes of the experiment. UV-VIS absorption and circular dichroism spectra were measured in the range 310-550 nm and 260-550 nm, respectively. Excitation and emission wavelengths used for fluorescence measurements were 370/440 and 335/385 nm for auto-fluorescence (Total AGEs) and pentosidine, respectively.

UV-VIS absorption spectra of Serva preparation showed all characteristics typical for PLP form of coenzyme bound to AST, i.e. absorption maximum at 360 nm and smaller shoulder at 430 nm. Pyridoxamine (PMN) form of coenzyme is formed upon reaction with L-aspartate. It is characterized by the shift of absorption maximum to 330 nm. Absorption profile of the Sigma-Aldrich preparation is similar to that of Serva (pyridoxal form) just flatter. Roche preparation has absorption maxima at 330 nm, which reminds PMN form or free PMN. Pyridoxal form of coenzyme was partly restored after dialysis.

Characteristics of pyridoxal as well as pyridoxamine forms of the coenzyme bound to AST, which are described in the literature, are the same as at absorption spectra. AST preparations obtained from Serva and Roche exhibit typical spectra of PLP form while Sigma preparation shows lower positive peak at 360 nm and additional plateau between 440 and 510 nm, which is unrepresentative for CD spectra of pure AST. CD spectra of AST obtained from Roche proved that PLP form prevails also in this preparation and the PMN form present at the absorption spectra is probably caused by free PMN, which is not optically active and that is why it is cleared away at CD spectra.

Fluorescence of Serva and Sigma samples is low and similar to that of the standard BSA, while fluorescence of Roche preparation is about two times greater. Fluorescence of Roche preparation decreases to some extent after dialysis, which suggests it could be caused by the low-molecular fluorescent compounds present in the sample before dialysis.

Serva preparation was chosen for future experiments because of its high fidelity of absorption and CD spectra together with low fluorescence. The enzyme obtained from Roche could be used for measurements of catalytic activity but it is not suitable for fluorescence measurements during glycation studies.

5.2. Glycation of AST by D-fructose *in vitro*

Boušová, I., Vukasović, D., Juretić, D., Palička, V., Dršata, J. (2005) Enzyme activity and AGE formation in a model of glycoxidation of AST by D-fructose in vitro. Acta Pharm. 55: 107-114.

D-Fructose is the most common, naturally occurring monosaccharide of human diets. Concentrations of fructose in the tissues with active polyol pathway are of the same order of magnitude as that of glucose and are strongly increased by hyperglycemia, which makes *in vivo* glycation by fructose a highly probable event. Apart from the extracellular glycation of long-lived proteins, the rapid intracellular AGEs formation by various intracellular precursors has been described. Those precursors are represented by fructose and its metabolites glyceraldehyde, dihydroxyacetone-phosphate, glyceraldehyde-3-phosphate and the α-dicarbonyl compounds (MGO and 3-DG). These compounds are believed to be important intracellular precursors of *in vivo* formed AGEs. Several *in vitro* studies carried out in buffer systems showed that fructose may serve as a precursor for pentosidine (Schalkwijk et al. 2004).

D-Fructose has been used as a glycating agent in the glycation studies already performed by our research group (Beranek et al. 2001; Beranek et al. 2002; Drsata et al. 2002). Alanine aminotransferase or aspartate aminotransferase served as model proteins in these studies. Aspartate aminotransferase has been chosen as a model protein for future experiments because of its higher stability during long-term incubations with a glycating agent. The aim of the presented study was to show the possible applicability of this model for monitoring the oxidation part of the glycoxidation process through measurements of fluorescent AGEs and pentosidine. The other criterion of protein modification was enzyme activity, which has been employed also in our previous glycation studies.

Incubation mixtures containing AST 1.33 mg/ml and 50 mM D-fructose (final concentrations) were incubated for up to 21 days at 37°C. The rising fluorescent AGEs were measured using 2 specific excitation and emission wavelengths corresponding to the Total AGEs ($\lambda_{exc}/\lambda_{em}$ 370/440 nm) and pentosidine ($\lambda_{exc}/\lambda_{em}$ 335/385 nm). Bovine serum albumin was used as reference compound for fluorescence measurements in concentration 1 mg/ml.

The enzyme activity of the incubation mixture containing AST only (control sample) was stable during the whole course of experiment, e.g. 21 days. The day-by-day fluctuations were caused mostly by daily setup of the assay in the laboratory. The presence of 50 mM D-fructose in the incubation mixture caused significant decrease in enzyme activity, e.g. to 42% and 11% of the control value on the fifth and last day of incubation, respectively.

Samples for fluorescence measurements were incubated for 15 days only. Generation of Total AGEs by the effect of fructose was increased about 45% on the fifteenth day in comparison with the control sample. Increase of pentosidine fluorescence was slower and less expressed than the fluorescence of Total AGEs. Pentosidine formation was increased about 20% on day 15 comparing to control sample.

The proposed model of glycation is efficient enough for the investigation of fructose-induced modification of AST and its affection by antioxidants using the measurement of enzyme activity and fluorescence of AGEs. Furthermore, generation of fluorescent AGEs can be enhanced by addition of transition metal ions (Cu^{2+} and Fe^{3+}) into the incubation mixtures. The obtained data have not been published yet, though they have been presented at several symposia (Boušová, I. et al.: National Congress of Clinical Biochemistry, 2005, Olomouc, Czech Republic; Dršata, J. and Boušová, I.: International Congress of FIP, 2006, Salvador Bahia, Brazil).

5.3. Evaluation of a possible antiglycation effects of several phytochemical components using *in vitro* model of glycation

Boušová, I., Martin, J., Jahodář, L., Dušek, J., Palička, V., Držata, J. (2005) Evaluation of in vitro effects of natural substances of plant origin using a model of protein glycoxidation. J Pharm Biomed Anal. 37(5): 957-962.

Flavonoid baicalin and its aglycon baicalein occur naturally in the plant *Scutellaria baicalensis Georgii*. The dried roots of this plant are widely used in traditional Chinese herbal medicine as popular antibacterial and antiviral agents. Baicalin, the glycoside of baicalein, exerts good anti-inflammatory and anti-tumor activity. Baicalein is able to protect the organism against cardiovascular diseases and it is also a good radical scavenger (Huang et al. 2005). The active principles of the plant *Arctostaphylos uva-ursi* are phenolic glucosides, e.g. arbutin. Leaves of this plant are used in herbal medicine for their disinfection effect on the urinary tract (Jahodar et al. 1978). Arbutin, a derivative of hydroquinone, seems to exert antiradical scavenging properties towards lipid peroxidation (Ioku et al. 1992). Hydroxycitric acid is the principal acid present in fruits of plants *Garcinia spp*. This compound has been described as a potent inhibitor of ATP citrate lyase in mitochondria, which participates in the metabolism of fatty acids. Thus, hydroxycitric acid can be used as dietary supplement for weight loss (Jena et al. 2002). The antioxidant and antiradical activities of this compound are not known. Hydroxycinnamic acids are the most widely distributed phenolic compounds in plant tissues. Their antioxidant and antiradical activities are described in detail in chapter 3.2.4.1.1.

Non-enzymatic glycation includes some oxidative steps and hence it is also called glycoxidation. Considering the importance of glycoxidation processes in the progress of diabetic pathology, antioxidants supplementation may be an important factor in the inhibition of protein modification, thereby preventing diabetic complications. The main purpose of the presented study was to evaluate the potential antiglycation effect of a group of natural phytochemical components with described antioxidant properties in the *in vitro* model of glycation. Chemical structures of the tested compounds are showed below (Figure 7).

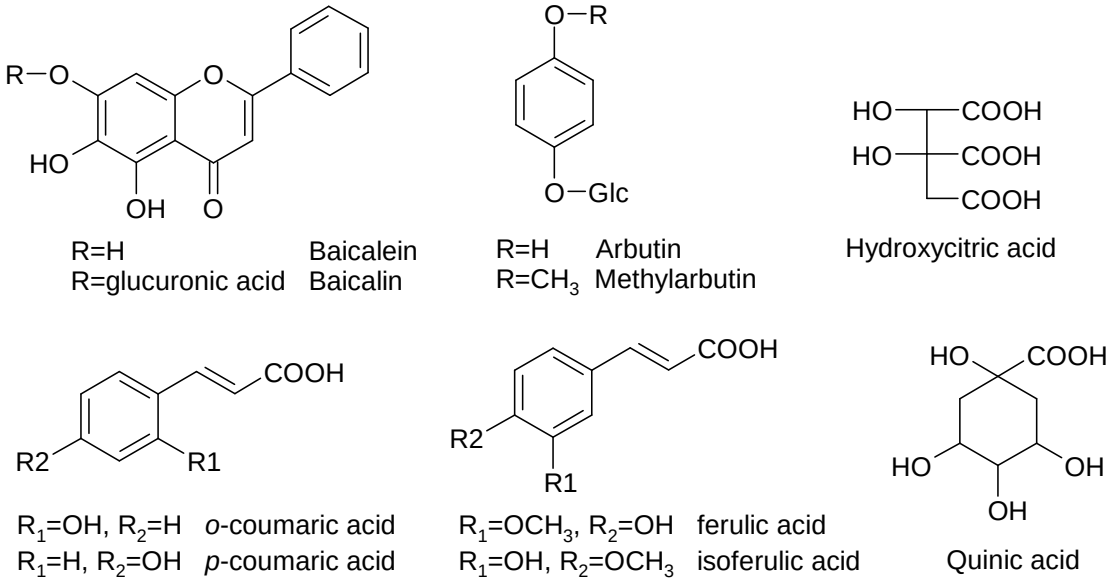


Fig. 7: Chemical structures of the studied phytochemical components

The direct antioxidant-protein interactions and indirect antiglycation effects of the tested compounds were observed. The phytochemical components were tested in the range of concentrations 0.5 – 50 mM. Enzyme activity was used as a measure of proceeding glycation. Samples were incubated at 37°C for up to 24 days.

The negative direct effects were probably caused by direct interaction of the antioxidant with the molecule of protein. These direct effects result in the decrease of AST catalytic activity. Not all tested substances had pronounced negative direct effects such as hydroxycitric, quinic, *o*-coumaric, and isoferulic acid. Compounds with highly pronounced direct effects were flavonoids baicalin and baicalein, and phenolic compounds arbutin, methylarbutin, ferulic and *p*-coumaric acid. The direct effects were dependent upon concentration as can be seen in the case of baicalin and baicalein. Hydroxycinnamic acids, arbutin and methylarbutin exerted negative direct effect only when tested in 50 mM concentration.

Several compounds (hydroxycitric, *p*-coumaric, *o*-coumaric acid, and arbutin) showed positive antiglycation influence mostly in lower tested concentrations. Two tested flavonoids as well as methylarbutin and ferulic acid did not show any positive influence on the glycation of AST throughout the experiment. Therefore, these substances can be considered as negatively acting compounds. Quinic and isoferulic acid showed neither significant positive nor negative influences, thus they can be regarded as neutral compounds. *O*-Coumaric acid can be indicated as a substance with overall positive effect.

Antioxidant activity of a group of hydroxycinnamic acids is strongly dependent on the number and position of hydroxyl groups. Hydroxyl group present in the *para* position is responsible for the antioxidant effect. Hydroxyl group present in the *ortho* or *meta* position has little or no effect. Presence of additional hydroxyl groups enhances the scavenging ability (e.g. caffeic acid). Substitution of *para*-hydroxyl group in caffeic acid by methoxyl group, as in the case of ferulic acid, causes a slight decrease of the antioxidant activity. Expected order of antioxidant activities of the tested HCAs is ferulic > *p*-coumaric >> *o*-coumaric acid. This order was confirmed only in the case of negative direct effects on the catalytic activity of AST. This suggests that structural features of HCAs responsible for scavenging activities are also closely connected with their reactivity with molecules that should be protected at the same time (proteins).

The model of AST glycation *in vitro* seems to be suitable also for testing antiglycation activities of natural antioxidants. Enzyme activity is a good parameter for monitoring the proceeding glycation as a measure of protein molecule impairment.

5.4. Antioxidative effect of uric acid towards glycation-induced modification of AST

Boušová, I., Bakala, H., Chudáček, R., Palička, V., Dršata, J. (2005) Glycation-induced inactivation of aspartate aminotransferase, effect of uric acid. Mol Cell Biochem. 278(1-2): 85-92.

Uric acid was, for a long time, considered to be only a waste product of the purine degradation without any physiological significance. Nowadays, the opinion that uric acid is an important endogenous antioxidant is generally accepted. This compound is able to scavenge hydroxyl radicals and hypochlorous acid (Becker 1993). It is also capable of an inhibition of iron-catalyzed oxidation of ascorbate in biological fluids (Davies et al. 1986; Sevanian et al. 1991). A negative feature of uric acid is its low solubility in water and its potential to form crystals in the vicinity of joints or concrements in the urinary tract. Lack of data in literature that would describe the influence of uric acid in the process of non-enzymatic glycation was the impulse, which leaded to the start of this work.

The main goal of this work was to investigate possibilities of uric acid intervention in the glycation-induced inactivation of aspartate aminotransferase in our model of glycation (described above). The experiments were carried out in co-operation with Laboratoire de Biologie et Biochimie Cellulaire du Vieillissement, Université Paris 7 – Denis Diderot, Paris, France.

D-Fructose in two concentrations (50 mM and 500 mM) was used as a glycating agent. Uric acid was tested in the range of concentration 0.2 – 1.2 mM. Incubation mixtures were incubated for up to 25 days at 37°C. Four different parameters were used to determine the effect of uric acid: catalytic activity of AST, generation of fluorescent AGEs, isoelectric point (pI) of glycated AST, and molecular charge of AST.

Uric acid in concentrations 0.2 - 0.6 mM exerted neither harmful direct nor positive antiglycating effects in preliminary experiments and therefore only 1.2 mM uric acid was tested in all following experiments. This compound itself did not affect the enzyme activity and it did not cause any increase in the formation of AGEs throughout the experiment. However, the molecule of AST was protected by uric acid against the deteriorating effects of D-fructose, which caused marked decrease in its

catalytic activity and rise of fluorescent AGE products formation (total AGEs and pentosidine).

In the course of glycation, basic amino groups of lysine and arginine loose their basic character upon the reaction with D-fructose. Theoretically, the molecule of the glycated protein becomes more anionic (negatively charged) and its pI shifts down to acidic pH. These phenomena were examined by physicochemical analysis (isoelectric focusing and native PAGE electrophoresis). In the course of our experiments, the molecule of AST became more anionic due to glycation by 500 mM D-fructose. This event was inhibited by uric acid present in the incubation mixture. The theoretical value of pI for AST is 6.80, but because four bands were found in our control sample we suppose four different isoforms of AST were present. Three out of four bands underwent the shift towards a more acidic pH within 25 days of incubation with D-fructose. The effect of D-fructose on AST pI was diminished by uric acid.

In conclusion, uric acid showed a remarkable inhibitory effect in the process of advanced nonenzymatic glycation. The fact that uric acid inhibited the formation of fluorescent AGE adducts suggests that this compound could at least in part trap α-dicarbonyl intermediates in AGEs formation. We would like to further investigate this possibility in future experiments.

5.5. Oxidative stress parameters in different systemic rheumatic diseases

Firuzi, O., Fuksa, L., Spadaro, C., Boušová, I., Riccieri, V., Spadaro, A., Petrucci, R., Marrosu, G., Saso, L. (2006) Oxidative stress parameters in different systemic rheumatic diseases. J Pharm Pharmacol. 58(7): 951-957.

Systemic sclerosis (scleroderma), osteoarthritis, rheumatoid arthritis, systemic lupus erythematosus, and psoriatic arthritis belong to a group of rheumatic disorders, which are autoimmune diseases characterized by destruction of cartilage and connective tissue. These diseases have two things in common: they cause chronic pain and they are difficult to treat. Lower levels of antioxidants and increased lipid peroxidation in plasma and synovial fluid of patients suffering from rheumatic diseases have been reported (Sarban et al. 2005; Taysi et al. 2002). Oxidative stress has been proposed as one of the factors contributing to the inflammation and further damage of the joints (Halliwell 1995).

The role of free radicals in the pathogenesis of rheumatic diseases has been studied in this work. Changes in plasma levels of four parameters of oxidative stress in patients with rheumatoid arthritis, systemic sclerosis, and psoriatic arthritis were assessed and the obtained values were compared with the levels found in healthy subjects. The measured parameters were total antioxidant capacity of plasma and concentrations of hydroperoxides, sulfhydryl groups, and carbonyl groups.

A group of thirty-six patients with rheumatic diseases was studied and the results were compared to a control group of twenty-two healthy subjects. The group of patients with rheumatic diseases was divided into 3 subgroups according to individual diseases: systemic sclerosis (17 patients), rheumatoid arthritis (9 patients), and psoriatic arthritis (10 patients). Venous blood was collected into EDTA-coated and sodium citrate-coated tubes according to requirements of the applied method. The employed assays were FRAP method assessing total antioxidant capacity, FOX method measuring hydroperoxides (e.g. products of lipid peroxidation), and assays for determination of sulfhydryl and carbonyl groups, both reflecting oxidation of proteins.

Markedly elevated levels of plasma hydroperoxides were found in all three groups of patients compared to the control group of healthy volunteers. Plasma of patients suffering from rheumatoid arthritis contained the highest levels of hydroperoxides. The level of carbonyls was significantly increased only in rheumatoid arthritis patients. The amount in carbonyl residues in psoriatic arthritis and systemic sclerosis patients did not differ from the control group. Plasma levels of sulfhydryl groups were decreased in all three groups of patients compared to control group. The lowest levels were found in rheumatoid arthritis. No differences in total antioxidant capacity of plasma between groups of patients and control group were found. The obtained data support the hypothesis that oxidative stress participates in the etiology of rheumatoid arthritis as well as in systemic sclerosis and psoriatic arthritis.

At least two out of four methods employed in this study seem to be suitable for investigation of protein glycoxidation *in vitro*. Introducing methods for assessing sulfhydryl and carbonyl groups in our future studies of protein glycation would be an important contribution to the understanding of this process.

6. CONCLUSIONS

1) The model of *in vitro* glycation of aspartate aminotransferase underwent several changes and improvements during the preparation of this thesis. Finally, the following model of AST glycation was established and used for investigation of a role of antioxidants in the process of glycation: a) control sample (AST only); b) control with glycating agent (AST + Frc 50 mM); c) AST + individual antioxidants (direct antioxidant-protein interactions); and d) AST + glycating agent + individual antioxidants (antiglycation effects of antioxidants).

    i) Several commercially available preparations of AST were tested using different spectroscopic methods (fluorescence, UV absorption and CD spectra). Based on the data obtained, the preparation supplied by Serva GmbH (Germany) was chosen as source of enzyme for the following experiments. This preparation exerted high fidelity of absorption and CD spectra together with its low fluorescence.

    ii) D-Fructose in 50 mM concentration was used as the glycating agent in our experiments. This sugar has sufficient glycating activity in the concentration used. Copper and iron ions were added to the incubation mixtures in order to support oxidative part of glycation process (data presented at conferences).

2) The palette of methods for monitoring the course of glycation and formation of advanced glycation end-products was extended.

    i) Fluorescence detection for pentosidine and non-specific AGEs (total AGEs) formation was successfully introduced. Total AGEs and pentosidine were measured at excitation and emission wavelengths 370/440 nm and 335/385 nm, respectively.

    ii) Four different methods for the evaluation of oxidative stress in patients with rheumatic diseases were applied. Two of them can be useful tools for the investigation of protein glycation as well as the intervention of antioxidants in this process.

3) Natural antioxidants expressed negative direct effects on the enzyme activity and positive antiglycation effects to various extents.

    i) The negative effects on the enzyme activity in incubation mixtures containing AST and individual antioxidants was probably caused by direct

interaction of the antioxidant tested with the molecule of protein. These effects were concentration-dependent. Not all tested antioxidants showed negative direct influence, namely uric acid, hydroxycitric acid, quinic acid, and *o*-coumaric acid exerted almost no direct interaction. By contrast, baicalin, baicalein, methylarbutin, and ferulic acid exerted remarkable direct interaction with the molecule of protein. It can be concluded that compounds with more pronounced antioxidant properties showed also more pronounced negative direct interactions with the molecule of enzyme.

ii) Antiglycation influence of the individual compounds was caused by the intervention of the compound in the process of glycation. Uric acid exerted a certain antiglycation effect. It protected AST against the loss of catalytic activity and changes in its physicochemical properties caused by the glycating agent, and partly inhibited the formation of fluorescent AGEs. Also some antioxidants of plant origin showed protective effects against protein glycation, e.g. *o*-coumaric acid, arbutin, and hydroxycitric acid.

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8. SUPPLEMENTS

Copies of published articles related to the topic of this doctoral thesis (I-V)

Abstracts of presentations and posters

List of abbreviations

I.

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Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 1173–1177
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Short communication

Determination of quality of pyridoxal-5′-phosphate enzyme preparations by spectroscopic methods

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Abstract

The present study evaluates purified aspartate transaminase (AST, EC 2.6.1.1) preparations from three commercial sources. The enzyme molecule contains pyridoxal-5′-phosphate coenzyme (PLP), which provides AST characteristic absorption spectra in the wavelength range of 300–500 nm. The coenzyme bound in the active site also shows circular dichroism (CD) spectra in the same range. Besides, AST like other proteins may be modified in vitro or in vivo by reactions with other molecules, e.g. reactive sugars, and may form fluorescent products (advanced glycation end products, AGE). Spectroscopic methods were used to assess the quality of AST preparations from three different sources, Serva, Roche, and Sigma. Absorption spectra showed that the peak 360 nm characteristic of the active PLP form of AST prevailed in the Serva and Sigma preparations, while 330 nm was the major peak in the Roche preparation. CD spectra demonstrated the major maximum at 360 nm in the Serva and Roche samples, thus suggesting the predominance of the active PLP form in both preparations. The Sigma sample showed a CD profile less characteristic of AST. Fluorescence measurements revealed formation of AGE in the case of the Roche preparation, while fluorescence of the other two preparations was low. In general, the Serva sample presented the most convenient properties of purified AST among the preparations tested. The results will be used for the selection of a commercial enzyme preparation applicable in our future spectroscopic studies of glycation of AST as a model protein and in our research of the influence of antioxidants on this process.

Keywords: Aspartate transaminase; Absorption spectra; Circular dichroism; Pyridoxal-5′-phosphate; Fluorescence; Protein purity

1. Introduction

Aspartate transaminase (AST, EC 2.6.1.1) is an enzyme frequently assayed in clinical laboratories. AST belongs to a group of enzymes characterized by the presence of pyridoxal-5′-phosphate (PLP) and its participation in catalysis. As for the protein part of the molecule, AST is composed of two identical, non-covalently bound subunits with more than 400 amino acid residues and possesses a molecular mass of about 93,000 Da, with some differences according to the animal species [1].

The PLP coenzyme is covalently bound to the ε-amino group of lysine 258 in an active site of aminotransferase [1–3]. It provides the AST molecule characteristic absorption spectra that differ from that of free PLP and which in general are the same regardless of species or cellular fraction (see Fig. 1 for an example).

The PLP form of AST shows, depending on pH, a major absorption peak at 360 nm (an active, unprotonized form of the coenzyme, prevailing at lower pH values [2]), and/or a peak at 430 nm (an inactive, protonized form, increasing at lower pH values). After a reaction with L-aspartate

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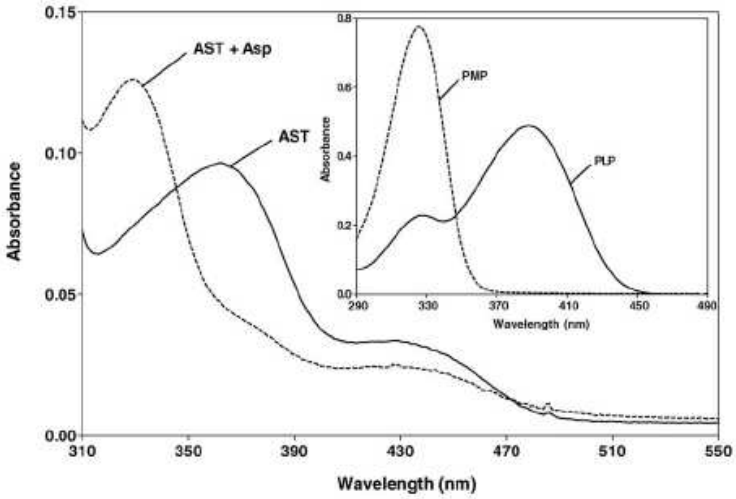

Fig. 1. Characteristic UV–vis absorption spectra of AST (Serva) and of the free coenzyme. Protein concentration 1.0 mg/ml of 0.1 M Na phosphate buffer pH 7.4), — PLP form (free AST); - - - PMP form (AST + Asp 1 mM). The insert shows the UV–vis spectra of free PLP (0.1 mM) and PMP (0.1 mM).

during the first part of a ping-pong transaminating reaction, the pyridoxamine-5′-phosphate (PMP) form of the coenzyme appears and the original absorption maxima shift to 325–330 nm.

While free PLP or PMP are not optically active substances, the coenzyme bound in the active site of AST shows circular dichroism (CD) spectra in the range of 300–500 nm, which are similar to absorption spectra. The CD effect is caused by the change in the electronic configuration of the molecule [4]. Circular dichroism clears away absorption characteristics of optically inactive components, which facilitates identification of the specific coenzyme signal and its changes, and also permits to uncover peaks of aromatic amino acids at 260–280 nm. Evaluation of intensity, characteristics of and changes in absorption and CD spectra facilitate, beside measurements of its catalytic activity, to assess the quality of enzyme preparations and help to understand the interactions of AST with other molecules.

Another property of proteins is their susceptibility to modify chemical reactions in vitro and in vivo. Such a typical reaction is the formation of advanced glycation end products (AGE), the process known in food chemistry for a century [5] and in medical biochemistry for decades [6]. AGE production accompanies diabetes mellitus and ageing and may indicate the history of interactions of individual protein molecules with sugars and other reactive compounds. Products of such protein modifications have more or less characteristic fluorescent properties. Fluorescence emission at 440 nm after excitation by light of 370 nm is considered to quantify total AGEs, while 335 nm excitation and 385 nm emission is partly specific for pentosidine as one of characteristic AGE products. The method is commonly used to evaluate haemoglobin glycation in the laboratory diagnosis and monitoring of diabetes.

We found out that the spectral as well as catalytical properties of AST make the enzyme a useful tool in drug–protein interaction [7] and in protein glycation studies [8]. It is advisable to have the enzyme as pure as possible for such research.

The enzyme is available in a relatively pure and stable form from several commercial sources and despite the fact that the commercial preparations are probably not intended for a special use in spectral studies, they appeared more or less convenient for our previous research [7]. The aim of the present study was to exploit the spectral properties and fluorescence of purified AST preparations from three sources for the evaluation and selection of preparations suitable for our future studies of protein glycation and the influence of antioxidants on this process.

2. Experimental

2.1. Chemicals

Cytosolic AST preparations from the porcine heart were obtained as suspensions in ammonium sulphate with properties partly declared:

(1) Serva Electrophoresis GmbH, Germany: Glutamate–oxaloacetate transaminase from the porcine heart in saturated ammonium sulphate, with 0.0025 M α-ketoglutarate, catalytic activity 291 U/mg;
(2) Roche Diagnostics GmbH, Germany: Glutamate–oxaloacetate transaminase (GOT) from the pig heart (catalytic activity not specified);

(3) Sigma–Aldrich GmbH, Steinheim, Germany, USA: Glutamate–oxaloacetate transaminase type I from the porcine heart. Suspension in 3.0 M $(NH_4)_2SO_4$ solution containing 0.05 M maleate and 2.5 mM α- ketoglutarate, pH 6.0, catalytic activity 284 U/mg.

Aspartic acid (p.a.) and sodium azide (p.a.) were obtained from Lachema, Brno, Czech Republic. Sodium phosphate buffer (0.1 M) pH 7.4 with 0.05% sodium azide (to prevent microbial contamination during experiments) was prepared from sodium hydrogen and dihydrogen phosphates and used for the preparation of all samples.

Protein contents of individual preparations and protein concentrations in samples prepared were checked by Bradford's method with Coomassie Brilliant Blue G-205 (CBBG) [9], [10] and calibrated by the bovine serum albumin (BSA) standard (Bio-Rad).

2.2. Sample preparation and incubation

All three suspensions of AST were centrifuged at 5000 rpm at 4 °C for 20 min. The supernatant was removed and enzyme pellets were reconstituted in an appropriate amount of 0.1 M sodium phosphate buffer pH 7.4 with 0.05% sodium azide in order to obtain solutions with protein concentration at 2.0 mg/ml for the preparation of final samples: AST 1 mg/ml in the buffer only (supposed PLP form of AST) and AST 1 mg/ml in buffer incubated 4 h with 1 mM L-aspartate (final concentrations). For some measurements, the samples prepared as above were dialyzed against sodium phosphate buffer and 0.05% sodium azide (three times for 1.5 h at 4 °C). All samples prepared were stored overnight in the dark at room temperature before the spectra were measured. In the case of dialysis, dialysis of selected samples was carried out against 200 ml of the standard buffer in a refrigerator (4 °C) three times for 90 min.

2.3. Absorption and circular dichroism spectra measurements

Absorption spectra were measured on a spectrophotometer HP 8453 in a 0.5 cm quartz cuvette against sodium phosphate buffer (pH 7.4; 0.1 M) with 0.05% sodium azide. Circular dichroism was measured on a dichrograph CD6 (Jobin Yvon, France, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague) in a 0.5 cm quartz cuvette against sodium phosphate buffer (pH 7.4; 0.1 M) with 0.05% sodium azide. The CD spectra were expressed as differential dichroic absorption $\Delta\varepsilon$.

2.4. Fluorescence of AST samples

The samples prepared by reconstitution of the enzyme solution from the precipitates were stored overnight at a dark room. Fluorescence was measured five times in separate samples (200 μl each) prepared from the same enzyme source on a luminescence spectrometer Perkin–Elmer LS 50B. Excitation and emission wavelengths: at 370/440 and 335/385 nm for total AGEs and pentosidine, respectively. The final results were expressed as relative fluorescence. Fluorescence of 200 μl of the BSA sample 1 mg/ml was taken as one arbitrary unit (AU).

3. Results and discussion

3.1. Absorption spectra of AST preparations

Fig. 2 shows the UV–vis absorption spectra of all three preparations. While the samples of Serva represent the typical absorption profile of present PLP coenzyme, and the Sigma profile is similar but flatter in the range of 310–470 nm, an absorption maximum about 330 nm prevails in the Roche preparation, reminding the PMP enzyme or free PMP.

Addition of L-aspartate to the Roche preparation caused a further increase in the maximum of 330 nm and disappearance of the shoulder at 360 nm, which confirmed formation of PMP form of the coenzyme from the original PLP form. On the other hand, the peak at 330 nm decreased after dialysis by one-third of its original height before dialysis, which demonstrates that at least a part of this peak was not caused by the firmly bound coenzyme.

3.2. Circular dichroism spectra of AST preparations

Fig. 3 represents characteristic CD spectra of the Serva preparation. Dialysis did not change the major positive peak, demonstrating thus the firmly bound PLP coenzyme. Addition of L-aspartate (1 mM) shifts this peak to 330 nm as a proof of transaminase activity.

An increase in CD signal at 260–290 nm suggests also a change in the aromatic amino acid region during the change in the active site. This change could not be observed on absorption spectra because absorption of aromatic amino acids of AST is drowned in the shoulder of the big peak caused by absorption of peptidic bonds of the protein.

Comparison of CD spectra of all three preparations (Fig. 4) demonstrates nearly identical properties of original PLP forms of the Serva and of Roche samples. This profile shows, above all, that the active PLP form prevails also in the Roche preparation.

Both preparations exhibit the same properties in the range of CD spectra of aromatic amino acids, too. The Sigma preparation of AST displays a remarkably lower positive peak of the active coenzyme at 360 nm, which agrees with the absorption profile of the sample in Fig. 2. An additional plateau between 440 and 510 nm is rather surprising and unrepresentative for CD spectra of relatively pure AST. The presence of 2-oxoglutarate in the preparation should, according to our experience [7], confirm the CD profile of PLP form of AST, an influence of maleic acid, declared in description of the preparation, should be demonstrated. The

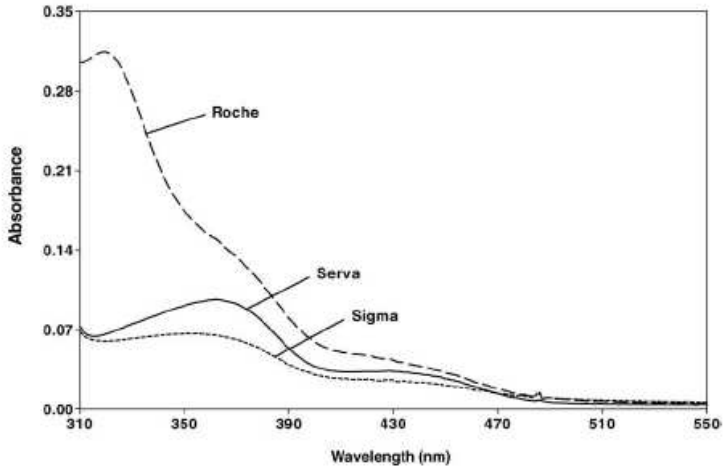

Fig. 2. UV–vis spectra of AST. Comparison of three preparations. Protein concentration 1.0 mg/ml of 0.1 M Na phosphate buffer pH 7.4. — Serva; –– Roche; - - - Sigma.

CD signal of aromatic amino acids is similar as for the profile but higher than with the Serva and Roche preparations.

3.3. Fluorescence

Table 1 summarizes the results of measurement of fluorescence of individual AST preparations.

While the fluorescence of the Sigma and Serva samples is similar to that of the standard BSA, the fluorescence of the Roche preparation is about twice of it. This suggests that the preparation contains molecules partly modified by reactive compounds like sugars. Dialysis had no remarkable influence on the fluorescence of the Serva and Sigma samples; the fluorescence of the preparation Roche partly decreased after dialysis, which suggests the presence of low-molecular fluorescence components beside the modified enzyme itself in the Roche preparation. We suppose that the cause of it might be either old animals as the source of the enzyme or a modifying step during processing of the product. The preparation will be convenient for measurements of catalytical activity but not for glycation studies.

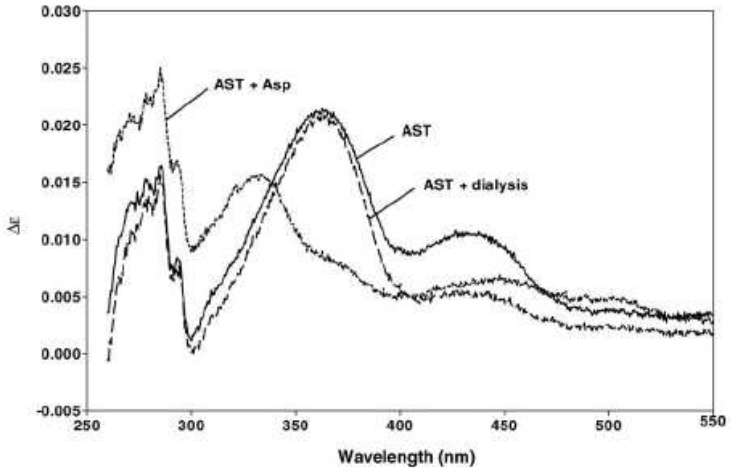

Fig. 3. Circular dichroism spectra of AST. (Serva Electrophoresis GmbH, Germany; protein concentration 1.0 mg/ml); protein concentration 1.0 mg/ml of 0.1 M Na phosphate buffer pH 7.4. — PLP form of AST; –– the same after dialysis; - - - PMP form (AST + Asp 1.0 mM).

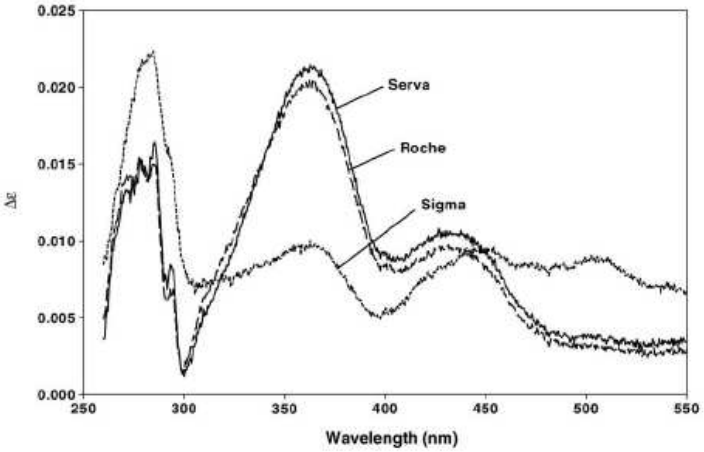

Fig. 4. Circular dichroism spectra of AST. Comparison of three preparations. Undialyzed samples. Protein concentration 1.0 mg/ml of 0.1 M Na phosphate buffer pH 7.4. — Serva; – – Roche; - - - Sigma.

Table 1
Fluorescence of three AST preparations

Preparation	Incubation sample[a]	Fluorescence (AU/mg)[b]	
		Total AGEs	Pentosidine
Serva	AST	0.83 ± 0.02	1.02 ± 0.13
	AST after dialysis	0.91 ± 0.01	1.13 ± 0.05
Sigma	AST	0.97 ± 0.05	1.16 ± 0.17
	AST after dialysis	0.94 ± 0.01	1.09 ± 0.05
Roche	AST	1.66 ± 0.03	1.81 ± 0.11
	AST after dialysis	1.12 ± 0.01	1.35 ± 0.10

[a] Each sample = 200 μl, protein concentration 1.0 mg/ml in 0.1 M Na phosphate buffer pH 7.4.
[b] Relative fluorescence of AST vs. BSA (BSA fluorescence = 1.0 AU). Each value = average ± S.D. of five separately processed and measured samples of the same preparation.

4. Conclusions

Among the AST preparations tested, Serva demonstrated the highest fidelity of absorption and CD spectra to the profiles described in the literature, and low fluorescence of the preparation. The preparation is suitable for application in any study of enzyme glycation or interactions with other molecules, e.g. drugs. The other two preparations differ more or less from the characteristic pattern, the Sigma sample having less characteristic CD spectra and the Roche preparation remarkable fluorescence.

Acknowledgements

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II.

Acta Pharm. 55 (2005) 107–114 Short communication

Enzyme activity and AGE formation in a model of AST glycoxidation by D-fructose *in vitro*

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Non-enzymatic glycation as the chain reaction between reducing sugars and free amino groups of proteins has been shown to correlate with physiological ageing and severity of diabetes. The process involves oxidative steps (glycoxidation). In this paper, the effect of D-fructose as a reactive sugar on aspartate aminotransferase (AST) as a model protein was monitored by measurements of the enzyme activity and formation of fluorescent advanced glycation end products (AGEs). Change in the AST activity was considered as a measure of the overall protein damage caused by glycation, and total AGEs and pentosidine represent, at least partly, the formation of glycoxidation products. Catalytic activity of AST in an incubation mixture containing D-fructose (50 mmol L^{-1}), decreased compared to control values to 42% ($p < 0.05$) and to 11% ($p < 0.05$) on the 5th and on 21st day of incubation, respectively. In the presence of fructose, total fluorescent AGEs concentration was significantly higher since 5th day of incubation (110%, $p < 0.05$) and the fluorescent pentosidine concentration from 15th day of incubation (117%, $p < 0.05$) compared to control values, respectively. Catalytic activity of AST clearly and quantitatively demonstrated functional changes in the enzyme molecule caused by structural modifications initiated by fructose, while the evaluation of AGE formation and especially that of pentosidine by fluorescence measurement was less reliable.

Keywords: non-enzymatic glycation, glycoxidation, aspartate aminotransferase, AGE, pentosidine

Reducing sugars (*e.g.*, glucose, fructose, galactose, mannose, ribose) and certain other carbohydrate relatives (*e.g.*, ascorbic acid) are inherently reactive toward nucleophiles (Maillard reaction) (1). Glucose can react with a free amino group (nucleophilic nitrogen base), such as an ε-amino group of protein lysine residue, to form an adduct commonly referred to a Schiff base. Formation of the Schiff base from sugar and amine is a fast and highly reversible reaction. Formation of an Amadori product (AP) from the Schiff base is

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slow but it is much faster than the reverse reaction, so the Amadori glycation tends to accumulate in proteins. The later stage, complex pigments and crosslinks have come to be known as advanced glycation end products (AGEs). AGEs constitute a heterogenous group of irreversible adducts that were originally characterized by their yellow-brown colour. It is known that these irreversible products include many protein-protein crosslinking structures as well as various non-crosslinked structures. Many, but not all of these products incorporate ultraviolet-visible and/or fluorescent chromophores (2).

There are three routes to producing AGEs: (*i*) via the autooxidative pathway (sugar gives reactive products by autooxidation) (3), (*ii*) via conventional Amadori rearrangement (4), and (*iii*) from the Schiff base (5). Reactive oxygen species: $O_2^{\bullet -}$, H_2O_2, $^{\bullet}OH$ (ROS) may contribute to these reactions, which require trace levels of catalytic redox-active transition metal ions. The process includes also oxidative steps and is therefore called glycoxidation (6).

Both glycation and oxidation reactions lead to the formation of AGEs, including the subclass of AGEs known as glycoxidation products (GOPs): pentosidine and N^{ε}-(carboxymethyl)-lysine (CML). The production of AGEs is accelerated by oxidative stress without the presence of sugar. There is evidence for a rise of pentosidine and CML from ascorbic acid (9).

Glycoxidation is considered to cause gradual deterioration in the structure and function of tissue proteins and to contribute to the pathophysiology of normal ageing. This post-translational modification of proteins and other macromolecules with amino groups is accelerated by hyperglycemia in diabetes, by hyperlipidemia in atherosclerosis, and by oxidative stress in other chronic diseases including Alzheimer's and Parkinson's disease (8–10). Furthermore, in diabetic patients with impaired renal function, food-derived AGEs with crosslinking activity can accumulate in serum to a significant degree, implying that normal kidney function is important for protection against dietary AGEs (11, 12). Another source of orally uptaken AGEs seems to be smoking, since significantly increased serum AGE levels have been observed in diabetic smokers compared to diabetic non-smokers (13).

It means that the level of AGE proteins reflects the kinetic balance of two opposite processes: the rate of AGE compounds formation and the rate of their degradation and elimination (14). This fact indicates the possibility of therapeutic intervention against AGEs by stimulation of their degradation or by inhibition of their formation (15). Further possibilities of therapy come from the knowledge of the character of protein modifications caused by glycoxidation (changes in their physico-chemical properties, such as solubility, isoelectric point, charge, ability of crosslinking, resistance to denaturation, *etc.*) (2).

Giardino *et al.* (16) demonstrated by *in vitro* experiments that one-week incubation of endothelial cells in the presence of a high glucose concentration resulted in a 13.8-fold increase in the intracellular AGE-content. In parallel, the mitogenic activity of endothelial cells cultivated at a high glucose concentration decreased markedly. The observed loss in mitogenic activity was due to the post-translational modification of the basic fibroblast growth factor (bFGF) by AGE, the major AGE-modification in endothelial cells.

In our laboratory, a model of aspartate aminotransferase or alanine aminotransferase as a protein and D-fructose as the glycating agent was developed and applied in several glycation studies (17, 18), in which enzyme activity served as the criterion of protein

modification. The objective of this study is to show the possible applicability of this model for monitoring the oxidation part of the glycation process in the presence of fructose through measurements of fluorescent AGEs and pentosidine.

EXPERIMENTAL

Chemicals

In vitro glycation was studied with aspartate aminotransferase (AST, EC 2.6.1.1), cytosolic enzyme from porcine heart, suspension in ammonium sulphate (SERVA Electrophoresis GmbH, Germany and Roche Diagnostics GmbH, Germany). Fructose was used as the glycating agent (Sigma-Aldrich Co. Ltd., USA). Other chemicals used, such as $NaH_2PO_4 \times 2H_2O$, $Na_2HPO_4 \times 12H_2O$, NaN_3, and uric acid, were of analytical grade. The reagent kit for enzyme activity measurements was purchased from Roche Diagnostics GmbH, Germany. Albumin, Bovine Fraction V, 96–99%, was used as the reference substance for fluorescence measurements (Sigma Chemical Company, USA).

Buffer, enzyme and fructose preparation

Phosphate buffer (0.1 mol L^{-1}, pH 7.4) with 0.05% sodium azide was used for the preparation of incubation mixtures.

Suspension of AST was centrifuged for 20 min at 5000 rpm (at 4 °C), the supernatant was removed and the protein pellet was reconstituted in 0.1 mol L^{-1} phosphate buffer pH 7.4 to yield the stock solution with the enzyme concentration of 2.66 mg mL^{-1}.

100 mmol L^{-1} stock solution of fructose was prepared in 0.1 mol L^{-1} phosphate buffer pH 7.4 with 0.05% sodium azide.

Preparation of incubation mixtures

Incubation mixtures for investigation of the catalytic activity of AST and for the study of AGEs and pentosidine formation were prepared according to Table I.

Final concentrations of substances in the incubation mixture for activity of AST were as follows: 1.33 mg mL^{-1} AST and 50 mmol L^{-1} fructose. All samples were incubated for

Table I. Incubation mixtures

Incubation mixture	Enzyme (mL)	Buffer (mL)	Fructose (mL)	Final volume (mL)
Activity of AST				
AST	0.208	0.208	–	0.416
AST + fructose	0.208	–	0.208	0.416
AGEs and pentosidine formation				
AST	1.56	1.56	–	3.12
AST + fructose	1.56	–	1.56	3.12

up to 21 days at 37 °C, and enzyme activity was measured in triplicate on days: 0, 1, 5, 8, 12, 15, and 21. Control values represent the catalytic activity of AST incubated without fructose for the same time.

Final concentrations of substances in the incubation mixture for AGEs and pentosidine formation were as follows: 1.33 mg mL^{-1} AST and 50 mmol L^{-1} fructose. All samples were incubated for up to 15 days at 37 °C. The amount of 600 μL of each sample was taken on days: 0, 1, 3, 5, and 15 and stored in a freezer at –80 °C. Fluorescence was measured in triplicate at the end of the experiment.

Catalytic activity measurement

AST catalytic activity was measured by the IFCC-recommended method (19), on an automated clinical chemistry analyzer Hitachi 917 (Japan).

Fluorescence measurements

The effects of glycation and glycoxidation were determined from the specific fluorescence intensity resulting from pentosidine and total AGEs level. Fluorescence measurements of glycated AST samples were monitored using a luminescence spectrometer Perkin-Elmer LS 50B (UK) at excitation (λ_{ex}) and emission (λ_{em}) wavelengths of 335/385 nm (optimum for AGE biomarker pentosidine) and 370/440 nm for the total AGEs level. Bovine serum albumin (BSA) was used as the reference substance in a concentration of 1 mg mL^{-1}. Relative fluorescence of pentosidine or total AGEs was expressed in arbitrary units per mg of protein (1 AU corresponding to the fluorescence of 1 mg $mL^{-1}$of BSA).

Statistics

Differences between means were assessed using the Mann-Whitney rank sum-test. The values $p < 0.05$ were considered statistically significant. SigmaStat program for Windows, version 2.0, Jandel Corporation (USA) was used for statistical analysis.

RESULTS AND DISCUSSION

In vitro glycation and glycoxidation were studied with AST. The enzyme was chosen as a model protein for its availability in a highly purified stable form (cytosolic porcine heart enzyme) and because of the presence of a few lysine residues in the molecule, which can undergo the glycoxidation process. Furthermore, Okada *et al.* (20) considered glycation to be one of the possible mechanisms for the existing variants of this enzyme in animal cells.

Effect of glycation on catalytic activity of AST

Effect of 50 mmol L^{-1} fructose as the glycating agent on the catalytic activity of AST was monitored for 21 days at 37 °C. Effect of glycation on lowering the enzyme activity was evident from the beginning of the incubation with fructose. The most rapid decrease

occurred from the 1st to the 5th day. Catalytic activity of AST decreased to 84% on the first day and to 42% ($p < 0.05$) on the fifth day compared to the control value, representing catalytic activity of AST incubated without fructose for the same time. Enzyme activity continued to decrease to 23% on the 12th day, and to 11% on the 21st (Table II).

Table II. Catalytic activity of AST in incubation mixture with and without fructose

Incubation mixture[a]	AST (μkat L^{-1})[b]						
	Time (day)						
	0	1	5	8	12	15	21
AST	6.09 ± 0.38	5.29 ± 0.34	4.33 ± 0.17	6.59 ± 0.03	5.47 ± 0.06	6.83 ± 0.15	5.23 ± 0.58
AST + 50 mmol L^{-1} fructose	5.94 ± 0.23	4.43 ± 0.34	1.82 ± 0.41[c]	2.55 ± 0.13[c]	1.26 ± 0.06[c]	1.43 ± 0.08[c]	0.58 ± 0.02[c]

[a] Enzyme concentration: 1.33 mg mL^{-1}.
[b] Average activity ± *SD*, $n = 3$.
[c] Significantly different from the samples without fructose measured on the same day ($p < 0.05$).

In this study, the authors' intention was to monitor glycation and glycoxidation processes through the changes in catalytic activity of AST as well as by detection of its final products (total AGEs and pentosidine). Due to previous results, which indicated that glucose or glucose-6-phosphate *in vitro* showed weak or no significant effects on the catalytic activity of AST and that the Heyns product derived from fructose (ketoses) was considerably more reactive than the Amadori product, fructose was used as the glycating agent (18, 21, 22). Protein glycation by fructose (fructation) was also suggested to a major determinant in diabetes complications, especially in cells with a hyperactive sorbitol pathway (23). Another advantage of the use of fructose as the glycating agent in this study was that the process of fructation is probably connected with a higher rate of AGEs production than the process of protein modification by glucose. Although fluorescence is a useful marker for detection of AGE formation, the fluorescent AGE crosslinks (represented by pentosidine) are thought to account for only one percent or less of the total crosslinking structures formed under physiological conditions (24). Thus, the major AGE structures responsible for protein-protein crosslinking *in vivo* are the non-fluorescent structures that have not yet been conclusively identified.

Effect of fructose on the production of AGEs

Table III shows the effect of fructose on the total AGEs formation during fifteen days of enzyme incubation at 37 °C. Compared to the control values, the fluorescence measured at λ_{exc} 370 nm/λ_{em} 440 nm was significantly higher in samples with fructose on the 5th (110%, $p < 0.05$) and the 15th day (145%, $p < 0.05$).

Table IV shows the effect of fructose on pentosidine formation during fifteen days of enzyme incubation at 37 °C. Compared to the control values, the fluorescence measured at λ_{exc} 335 nm/λ_{em} 385 nm was significantly higher in samples with fructose on the 15th day (117%, $p < 0.05$).

Table III. Fluorescence of glycated AST expressed as total AGEs (λ_{exc} 370 nm/λ_{em} 440 nm)

Incubation mixture[a]	Fluorescence (AU per mg of protein)[b,c]				
	Time (day)				
	0	1	3	5	15
AST	2.153 ± 0.075	2.231 ± 0.015	2.304 ± 0.034	2.186 ± 0.051	2.410 ± 0.030
AST + 50 mmol L^{-1} fructose	2.128 ± 0.015	2.212 ± 0.052	2.387 ± 0.011	2.465 ± 0.045[d]	3.511 ± 0.071[d]

[a] Enzyme concentration: 1.33 mg mL^{-1}.
[b] 1 AU corresponding to the fluorescence of 1 mg BSA mL^{-1}.
[c] Average fluorescence ± *SD*, $n = 3$.
[d] Significantly different from the samples without fructose measured on the same day ($p < 0.05$).

Table IV. Fluorescence of glycated AST expressed as pentosidine (λ_{exc} 335 nm/λ_{em}385 nm)

Incubation mixture[a]	Fluorescence (AU/mg per mg of protein)[b,c]				
	Time (day)				
	0	1	3	5	15
AST	2.472 ± 0.313	2.404 ± 0.073	2.513 ± 0.120	3.315 ± 0.092	3.733 ± 0.127
AST + 50 mmol L^{-1} fructose	2.323 ± 0.080	2.325 ± 0.074	2.459 ± 0.036	3.484 ± 0.247[d]	4.483 ± 0.337[d]

[a] Enzyme concentration: 1.33 mg mL^{-1}.
[b] 1 AU corresponding to the fluorescence of 1 mg BSA mL^{-1}.
[c] Average fluorescence ± *SD*, $n = 3$.
[d] Significantly different from the samples without fructose measured on the same day ($p < 0.05$).

According to the results, fructose induced a decrease in the catalytic activity of AST throughout the study period. A statistically significant decrease was observed even on the fifth day of incubation with fructose, while a substantial increase in fluorescence as the measure of total AGEs and pentosine formation was observed after 15 days.

CONCLUSIONS

This study presents the results of the measurements of fluorescent AGEs as representatives of the complex glycoxidating process. We can conclude that the proposed model is efficient enough for the investigation of AST modification by fructose using the measurement of the catalytic activity of the enzyme. In our future experiments with natural antioxidants, we are going to improve the AST-fructose model by introducing the transition metal ions (25, 26), which should support the oxidation part of the glycation process.

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SAŽETAK

Katalitička aktivnost i stvaranje AGE-produkata u modelu glikoksidacije AST D-fruktozom *in vitro*

IVA BOUŠOVÁ, DANKA VUKASOVIĆ, DUBRAVKA JURETIĆ, VLADIMÍR PALIČKA i JAROSLAV DRŠATA

Neenzimska glikacija je proces kojim se reducirajući šećeri kovalentnim vezama povezuju s amino skupinama proteina, bez posredovanja enzima. Taj proces dijelom je i oksidativan pa se često naziva glikooksidacija. Konačni produkti glikacije (AGE) nastaju polimerizacijom mnogih intermedijarnih produkata. U ovom radu, praćen je u *in vitro* uvjetima, mjerenjem katalitičke aktivnosti enzima i fluorescencije nastalih ukupnih AGE produkata i pentozidina, utjecaj D-fruktoze kao reaktivnog šećera na aspartat aminotransferazu (EC 2.6.1.1.) kao modelni protein. Promjena katalitičke aktivnosti enzima pokazatelj je promijenjene funkcije proteina glikacijom dok je intenzitet fluorescencije ukupnih AGE produkata i pentozidina samo djelomičan pokazatelj glikoksidacijskih promjena. Katalitička aktivnost AST izmjerena IFCC preporučenom metodom u inkubacijskoj smjesi koja je sadržavala 50 mmol L^{-1} fruktoze, smanjena je na 42% ($p < 0.05$) petog dana, te na 11% ($p < 0.05$) dvadeset i prvog dana. Statistički su značajne razlike u katalitičkoj aktivnosti enzima inkubiranog sa i bez fruktoze već nakon petog dana. Stvaranje ukupnih AGE produkata i pentozidina statistički je značajno nakon pet, odnosno 15 dana inkubacije enzima s fruktozom. Katalitička aktivnost enzima jasno ukazuje na funkcionalne promjene uzrokovane glikiranjem, dok je evaluacija stvaranja ukupnih AGE produkata, a pogotovo pentozidina, mjerenjem fluorescencije manje pouzdan pokazatelj promjena.

Ključne riječi: neenzimska glikacija, glikoksidacija, aspartat aminotransferaza, krajnji produkti glikacije, pentozidin

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Evaluation of in vitro effects of natural substances of plant origin using a model of protein glycoxidation

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Abstract

In an in vitro model with purified porcine aspartate aminotransferase (AST, EC 2.6.1.1) as the protein, the effects of phenolic antioxidants of plant origin (arbutin, methylarbutin, ferulic and isoferulic acids, *o*-coumaric and *p*-coumaric acids, quinic acid), flavonoids (baicalin and baicalein), and of hydroxycitric acid (HCA) at 0.5–50 mM concentration on the enzyme activities and on its glycation by 50mM D-fructose as the glycating agent were studied. During incubation with AST at 37 °C up to 24 days, fructose alone decreased AST activities as a result of protein glycation. In the absence of fructose, 50 mM phenolic compounds gradually decreased AST activity, while no or a weak effect of individual compounds was found at 3 mM concentration. A direct negative effect on AST was pronounced with ferulic acid. On the other hand, beneficial influences of phenolic compounds on glycation of AST by fructose were found mostly at 3 mM concentration. Effects on glycation were vague at 50 mM concentration, probably due to a combination of direct negative influences and antiglycation effects of individual compounds. No effect, neither positive nor negative, on AST activity and protein glycation, was found with quinic acid. The flavonoid baicalin and its aglycon baicalein rapidly decreased the in vitro activity of the enzyme in all concentrations used (0.5–3 mM), and no beneficial effects of the compounds on glycation of the enzyme by fructose were found. The influence of HCA on glycation was concentration-depended, ranging from beneficial inhibition of glycation at 2.5 mM concentration to a strong decrease in AST activity at 10 mM HCA. Both the beneficial and undesirable effects of natural antioxidants should be considered in case they are used as antiglycation factors. The results obtained can contribute to the evaluation of quality of various generally recommended antioxidants.

Keywords: Aspartate aminotransferase; Protein glycation; Phenolic antioxidants; Hydroxycitric acid; Flavonoids; Enzyme activity

1. Introduction

Glycation, a non-enzymatic reaction between free amino groups of proteins and reducing sugars, is closely associated with the pathogenesis of age- and diabetes-related complications like neuropathy, angiopathy and nephropathy [1]. This process represents a common posttranslational modification of proteins, which can impair their functions in living organisms. The oxidative steps are also involved and the process can be therefore called glycoxidation [2]. Free radicals, products of the autooxidation of the glycating sugar, and a heterogeneous group of substances called advanced glycation end products (AGEs) are formed in the course of glycoxidation [3].

Aspartate aminotransferase (AST, EC 2.6.1.1) is a very useful enzyme in clinical laboratory diagnostics. The AST

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molecule is composed of two identical, non-covalently bound subunits, with one molecule of the coenzyme pyridoxal-5′-phosphate bound to each subunit. Regardless of its metabolic role, the enzyme represents a suitable model for glycation studies [4], because the molecule contains 15–20 lysine residues (according to animal species) participating in the course of glycation. Glycation of AST by reactive monosaccharides (e.g. glucose, fructose) is accompanied by a decrease in its catalytic activity in dependence on the concentration and activity of the glycating agent [4,5].

With regard to the presence of free radicals and oxidative steps in the glycoxidation process, compounds with antioxidative effects have been tested in order to slow down or to stop glycoxidation [6–8]. The list include compounds like α-lipoic acid, α-tocopherol, ascorbic acid, β-carotene, aminoguanidine, pyridoxamine, which showed different antiglycation potential from no effect (α-tocopherol) to the effect at the dose 600 mg/day (α-lipoic acid). Description of antioxidant properties and their explanations are ambiguous.

Antioxidants may be divided into several groups according to several aspects (e.g. solubility, origin, and mechanism of action). Primary antioxidants inhibit the formation of free radicals (e.g. inhibitors of xanthinoxidase and NADPH oxidase, ion chelators); secondary antioxidants are able to scavenge the already formed free radicals (e.g. superoxid dismutase, substances with reducing activity); tertiary antioxidants repair or eliminate molecules damaged by free radicals [9]. The present study deals with secondary antioxidants of plant origin possessing reducing activity.

Baicalin and its aglycon baicalein are natural polyphenolic compounds belonging to the group of flavones, which occur in the plant *Scutellaria baicalensis Georgi*. These flavonoids have proven the following effects: antioxidative, immunomodulative, antiflogistic, antibacterial, antiviral, sedative, and partially cytostatic. Both substances are able to scavenge hydroxyl- and alkyl-radicals, and baicalein also traps superoxide anion radicals [10].

Hydroxycinnamic acids (e.g. caffeic, ferulic, coumaric) are widely distributed compounds in the plant kingdom, which usually exist as simple esters with quinic acid or glucose. Only carboxyl groups of hydroxycinnamic acids are included in the formation of these esters [11]. Hydroxycinnamic acids form highly resonance-stabilized radicals (phenoxy radical) after a reaction with reactive radicals, since the unpaired electron may be delocalised across the entire molecule. These phenoxy radicals are not able to initiate or propagate a radical chain reaction and their most probable fate is a collision and condensation with another radical [12].

Arbutin, a glucoside of hydroquinone, is found in the leaves of some medicinal plants (*Arctostaphylos uva-ursi*). Hydroquinone and its derivatives act as antioxidants by scavenging free radicals, and therefore, arbutin also seems to possess an antioxidative activity towards lipid peroxidation [13].

Hydroxycitric acid is a major metabolite in the fruit rinds of certain species of the plant *Garcinia* (*G. cambogia*, *G. indica*, *G. atroviridis*). This substance is at the centre of interest for its unique regulatory effect on fatty acid synthesis, lipogenesis, appetite, and weight loss [14]. Properties of hydroxycitric acid have been intensively studied in our laboratories using the tests of antiaggregation, cytotoxicity, and acute toxicity (results not yet published).

The aim of the present study was to evaluate a possible antiglycation effect of a group of the above-mentioned natural antioxidants of plant origin using our in vitro model of glycation.

2. Experimental

2.1. Chemicals

Aspartate aminotransferase, a cytosolic enzyme from the porcine heart suspended in saturated ammonium sulphate (291 U/mg), was obtained from Serva Electrophoresis GmbH, Germany. Baicalein and (−)-threo-hydroxycitric acid were purchased from Fluka Chemie, GmbH, Switzerland. Baicalin hydrate was obtained from Aldrich Chem. Co., Milwaukee, USA. Arbutin and methylarbutin were isolated from the leaves of *Arctostaphylos uva-ursi* at the Department of Pharmaceutical Botany and Ecology. The following compounds were products of Sigma Chemicals Co. (St. Louis, USA): ferulic acid, isoferulic acid, *o*-coumaric acid, *p*-coumaric acid, quinic acid, and D-(−)-fructose. Sodium azide was obtained from Lachema, Brno, Czech Republic. All chemicals were of analytical grade. Chemical structures of the compounds tested are presented in Fig. 1.

2.2. Sample preparation and incubation

The enzyme suspension was centrifuged at 5000 rpm at 4 °C for 20 min, the supernatant was removed, and the protein pellet was reconstituted in 0.1 M sodium phosphate buffer (pH 7.4, 0.05% sodium azide) to yield a stock solution of 2.66 mg/ml. After that, the enzyme solution was used for the preparation of four different types of incubation mixtures: (a) with buffer only (control samples), (b) with D-fructose (Frc) in a final concentration of 50 mmol/l (i.e. "protein glycation" samples), (c) with individual antioxidants in a final concentration of 0.5–50 mmol/l (i.e. "direct protein–antioxidant interaction" samples), (d) with individual compounds in a final concentration of 0.5–50 mmol/l and D-fructose in a final concentration of 50 mmol/l (i.e. "antiglycation" samples). The final concentration of the enzyme protein was 1.33 mg/ml. Incubation mixtures were incubated in the dark at 37 °C for up to 24 days.

2.3. Enzyme assay

The enzyme was assayed using the IFCC-recommended kinetic UV-method (Roche Diagnostics, Mannheim, Germany, an automatic analyzer Hitachi 917) [15]. Sampling

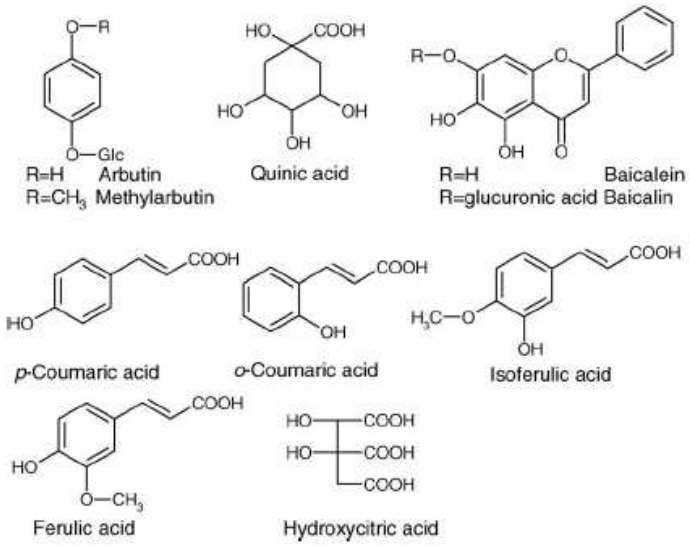

Fig. 1. Chemical structures of tested antioxidants.

and measuring was carried out on days 0–24, individual intervals were adopted according to the schedules of the clinical laboratory. Sample aliquots were diluted 1:2000 with 0.1 M sodium phosphate buffer (pH 7.4, 0.05% sodium azide) to obtain enzyme activities in the analytical range of the methods used. All experiments were carried out in triplicates. AST catalytic activity was calibrated by measurement of the standards Precinorm U and Precipath U (Roche Diagnostics, Germany) before and after each assay. The between-run coefficients of variation of the both assays for Precinorm U and Precipath U were lower than 2.0%.

2.4. Statistical analysis

Absolute values of enzyme activities (μkat/l) are given as the mean ± standard deviation (S.D.). Some presented data are expressed as the percentage of the respective control ± S.D. in order to avoid some day-to-day fading of enzyme activities and fluctuations caused by daily recalibrations of the analyzer. Statistical significance was determined by the use of Student's *t*-test and the differences were regarded as significant when $p < 0.05$.

3. Results and discussion

Most of tested compound had a more or less pronounced negative direct effect on enzyme activity, which was probably due to a direct interaction of the molecule of the antioxidant with the molecule of the enzyme. In addition, positive (indirect, antiglycation) effects were observed with some compounds.

A negative direct influence on enzyme activity was mostly pronounced in the case of baicalin (see Fig. 2).

This substance at 0.5 mM concentration caused a statistically significant decrease in enzyme activity already after five days of incubation. The direct effect of baicalin was dependent upon concentration (day 5: control sample 5.36 ± 0.04 μkat/l; baicalin 0.5 mM 3.97 ± 0.47 μkat/l; 1.0 mM 2.89 ± 0.05 μkat/l; 1.5 mM 2.42 ± 0.27 μkat/l;

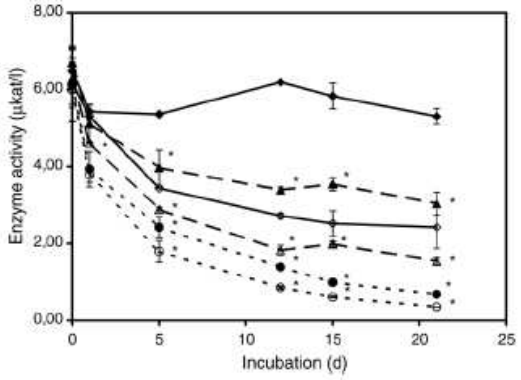

Fig. 2. Direct effect of baicalin on the AST activity in vitro. Concentration of AST in incubation mixtures was 1.33 mg/ml in 0.1 M sodium phosphate buffer (pH 7.4; 0.05% sodium azide). Incubation at 37 °C. The samples were diluted before the assay to fit to the analytical range of the method. Values are expressed as mean ± S.D. of six (control and AST + Frc 50 mM) or three (with baicalin) independent samples (*$p < 0.05$). (♦): Control sample (AST), (◊): AST + Frc 50 mM, (▲): baicalin 0.5 mM + AST, (△): baicalin 1.0 mM + AST, (●): baicalin 1.5 mM + AST, (○): baicalin 3.0 mM + AST.

3.0 mM 1.79 ± 0.28 μkat/l). Similar results were obtained in the case of baicalein (results are not presented).

Activities of AST samples containing baicalin or baicalein and fructose were compared with those of the samples of AST and fructose 50 mM, whose activity was taken as 100%. Both baicalin and baicalein deepened a deteriorating influence of sugar on enzyme activity (day 5: control sample 5.36 ± 0.04 μkat/l; baicalin 0.5 mM + AST + Frc 50 mM 3.09 ± 0.08 μkat/l) and had no beneficial effects on the glycation of enzyme by fructose (day 5: 3.44 ± 0.01 μkat/l). Concentration dependence was determined in both cases. The results are summarized in Table 1. Obviously, no antiglycation effect of baicalin and baicalein was found.

Flavonoids with the greatest antioxidant activity fulfil the following structural arrangements: the 2,3-double bond in combination with both the 4-oxo function and the 3-hydroxyl group in the C ring; the *meta*-5,7-dihydroxy arrangements in the A ring; the *ortho*-3′,4′-dihydroxy moiety in the B ring [16]. Baicalin and baicalein, according to above-mentioned structure–activity relantionships, would react as week antioxidants.

Fig. 3 shows direct influences of hydroxycitric acid on AST activity. The enzyme activities of samples with fructose demonstrate a remarkable decrease caused by sugar in comparison with a minor direct effect of hydroxycitric acid. The direct effect of the compound on AST was negligible in all tested concentrations (2.5 mM, 5.0 mM, 7.5 mM, and 10.0 mM). A statistically significant decrease in enzyme activity was observed only in a few individual samples (once at any tested concentration) and could be omitted.

Hydroxycitric acid at 2.5 mM concentration showed a statistically significant positive antiglycation effect on days 5–15 (see Fig. 4). The same antiglycation effect was not observed at higher concentrations (5.0 mM, 7.5 mM, and 10.0 mM). For example, samples with fructose and 5.0 mM hydroxycitric acid showed a nearly identical enzyme activity as AST samples with fructose alone. It seems that hydroxycitric acid at higher concentrations supports the negative influence of fructose on AST activity. In general, the positive effects of hydroxycitric acid are prevailing at lower concentration, contributing thus to several other positive effects of the compound, which are described in the Introduction.

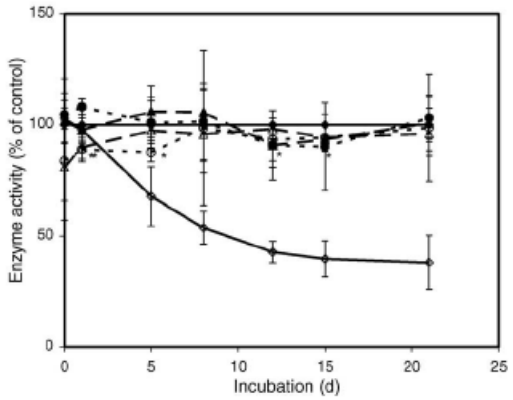

Fig. 3. Direct influence of hydroxycitric acid (HCA) on the AST activity in vitro. Values are expressed as a percentage of the activity of respective control (AST) ± S.D. (%) of six independent samples (*$p < 0.05$). (♦): Control sample (AST), (◊): AST + Frc 50 mM, (▲): HCA 2.5 mM + AST, (△): HCA 5.0 mM + AST, (●): HCA 7.5 mM + AST, (○): HCA 10.0 mM + AST. For other details see Fig. 2.

Phenolic antioxidants were tested at two different concentrations (3 mM and 50 mM). Table 2 summarizes direct effects of the group of phenolic antioxidants at 50 mM concentration on AST activity. Results obtained at 3.0 mM concentration are not presented, because the activity declined maximally by 8.3 ± 3.7% (ferulic acid) or apparently increased by 15.0 ± 5.1% (*p*-coumaric acid) in comparison with the control sample (100%), respectively. Quinic acid, *o*-coumaric acid, and isoferulic acid at 50 mM concentration had no effect on the AST activity and an apparent increase in enzyme activity was also observed. There is no exact explanation for

Table 1
Evaluation of antiglycation effect of baicalin and baicalein in enzyme model

Concentration	Incubation sample[a]	Enzyme activity (% of control)[c]		
		Day 5	Day 12	Day 21
	AST + Frc[b]	100.0 ± 0.3	100.0 ± 2.3	100.0 ± 13.1
0.5 mM	Baicalin + AST + Frc	89.9 ± 2.3	76.4 ± 1.8	41.7 ± 3.1
	Baicalein + AST + Frc	103.5 ± 2.6	61.4 ± 1.3	23.9 ± 3.2
1.0 mM	Baicalin + AST + Frc	74.7 ± 3.1	40.2 ± 4.1	15.3 ± 0.6
	Baicalein + AST + Frc	134.4 ± 55.2	52.5 ± 0.4	19.4 ± 0.9
1.5 mM	Baicalin + AST + Frc	61.4 ± 32.8	38.5 ± 10.1	11.6 ± 3.9
	Baicalein + AST + Frc	100.7 ± 3.4	74.0 ± 7.9	19.5 ± 1.3
3.0 mM	Baicalin + AST + Frc	62.3 ± 17.2	33.2 ± 5.6	9.1 ± 1.7
	Baicalein + AST + Frc	95.6 ± 21.1	47.3 ± 5.3	12.1 ± 1.3

[a] Protein concentration: 1.33 mg/ml in sodium phosphate buffer (0.1 M; pH 7.4) with 0.05% sodium azide. Incubation at 37 °C.
[b] Final concentration: 50 mM in all samples.
[c] Results are expressed as % of enzyme activity of the respective control (AST + Frc) ± S.D. Each value represents six (AST + Frc) or three (with baicalin or baicalein) individually prepared samples.

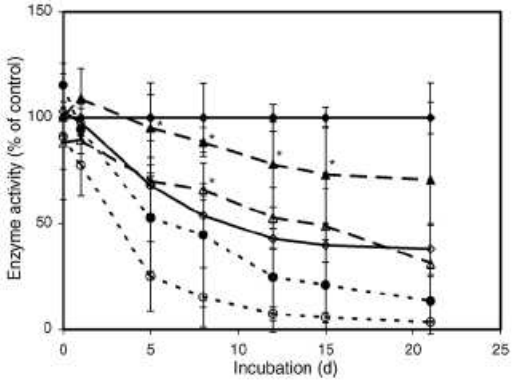

Fig. 4. The effect of hydroxycitric acid (HCA) on the glycation of AST by fructose in vitro. Values are expressed as a percentage of the activity of respective control (AST) ± S.D. of six independent samples ($^*p < 0.05$). (♦): Control sample (AST), (◇): AST + Frc 50 mM, (▲): HCA 2.5 mM + AST + Frc, (△): HCA 5.0 mM + AST + Frc, (●): HCA 7.5 mM + AST + Frc, (○): HCA 10.0 mM + AST + Frc. For other details see Figs. 1 and 2.

this increase. A statistically significant decrease in AST activity since the fourth day was observed in the case of arbutin, methylarbutin, *p*-coumaric acid, and ferulic acid at 50 mM concentration. The enzyme activity in the presence of the latter compound was the lowest among the phenolic antioxidants (see Table 2).

Antiglycation influences of the group of phenolic antioxidants at 3.0 mM and 50 mM concentrations are shown in Table 3.

Table 2
Direct effects of a group of phenolic antioxidants on AST activity

Incubation sample	Enzyme activity (% of control)[a]		
	Day 0	Day 4	Day 13
Control sample (AST)	100.0 ± 10.9	100.0 ± 8.2	100.0 ± 7.0
Arbutin 50 mM + AST	108.9 ± 5.4	79.1 ± 1.0*	79.9 ± 2.7*
Methylarbutin 50 mM + AST	104.2 ± 23.7	85.9 ± 1.8*	75.1 ± 7.7*
Quinic acid 50 mM + AST	99.5 ± 1.5	100.4 ± 8.7	97.1 ± 2.2
p-Coumaric acid 50 mM + AST	100.5 ± 2.7	93.6 ± 0.9*	50.5 ± 4.2*
o-Coumaric acid 50 mM + AST	103.7 ± 1.1	107.6 ± 2.3	110.0 ± 4.9
Ferulic acid 50 mM + AST	119.0 ± 2.5	47.4 ± 2.2*	10.5 ± 0.2*
Isoferulic acid 50 mM + AST	123.9 ± 3.0	112.8 ± 1.8	122.3 ± 10.0

[a] Results are expressed as % of enzyme activity of respective control (AST) ± S.D. (%). Enzyme activities of marked data (*) were significantly decreased in comparison with enzyme activity of control sample ($p < 0.05$). Each value represents six individually prepared samples. For other conditions see Table 1.

Table 3
Evaluation of antiglycation effect of a group of phenolic antioxidants in enzyme model

Incubation sample	Enzyme activity (% of control)		
	Day 0	Day 4	Day 13
AST + Frc 50 mM	100.0 ± 17.0	100.0 ± 3.2	100.0 ± 15.9
Arbutin 3 mM + AST + Frc	104.7 ± 0.5	123.8 ± 18.3*	115.4 ± 13.8
Arbutin 50 mM + AST + Frc	101.2 ± 1.9	139.0 ± 6.1*	121.9 ± 5.8*
Methylarbutin 3 mM + AST + Frc	95.1 ± 9.1	106.2 ± 2.4	102.5 ± 26.7
Methylarbutin 50 mM + AST + Frc	95.6 ± 0.9	91.9 ± 3.7	74.3 ± 10.5
Quinic acid 3 mM + AST + Frc	94.6 ± 7.8	100.5 ± 15.3	91.9 ± 46.5
Quinic acid 50 mM + AST + Frc	95.5 ± 1.4	72.9 ± 11.5	69.6 ± 31.9
p-Coumaric acid 3 mM + AST + Frc	96.0 ± 9.5	144.8 ± 29.6*	163.1 ± 28.7*
p-Coumaric acid 50 mM + AST + Frc	97.6 ± 1.7	180.4 ± 2.7*	81.1 ± 3.5
o-Coumaric acid 3 mM + AST + Frc	95.3 ± 13.0	142.9 ± 15.3*	140.0 ± 7.3*
o-Coumaric acid 50 mM + AST + Frc	106.6 ± 6.0	202.2 ± 9.1*	174.9 ± 4.3*
Ferulic acid 3 mM + AST + Frc	100.3 ± 14.9	113.6 ± 43.8	112.2 ± 1.1
Ferulic acid 50 mM + AST + Frc	112.9 ± 5.0	84.4 ± 5.2	23.4 ± 0.3
Isoferulic acid 3 mM + AST + Frc	101.3 ± 1.1	125.8 ± 36.1	161.9 ± 5.3*
Isoferulic acid 50 mM + AST + Frc	115.5 ± 4.9	108.2 ± 8.1	138.3 ± 1.6*

For conditions see Tables 1 and 2.

Quinic acid had no antiglycation effect at either concentration tested. Isoferulic acid at both concentrations showed a statistically significant influence on the glycation of enzyme only on the 13th day. These two above-mentioned compounds had neither a negative direct nor a positive indirect (antiglycation) effect and they may be considered as neutral. Since no beneficial effects were observed in the case of methylarbutin and ferulic acid and the compounds had negative direct influences, they can be regarded as negatively acting compounds. The only substance with a fully positive effect seems to be *o*-coumaric acid, which showed both no negative direct effect and a remarkable support of enzyme activity at both concentrations since the first day of the glycation experiment. Beneficial antiglycation effects outweighed the negative direct influences of arbutin (at both concentrations and throughout the experiment) and of *p*-coumaric acid (at 3 mM concentration between the 1st and 13th day, and at 50 mM concentration on days 1 and 4).

Antioxidant activity of hydroxycinnamic acids is mainly due to *para*-hydroxyl group, while there is little or no effect when OH group is present in the *meta* or *ortho* position. The presence of methoxy group (ferulic acid) enhances the electron donating properties in the *para*-position in comparison with monophenolics (*p*-coumaric acid). The expected or-

der of antioxidant activities of hydroxycinnamic acids is ferulic > *p*-coumaric ≫ *o*-coumaric [12,16,17]. This order was confirmed only in the case of negative direct effects on the enzyme activity, which suggests at least that the structural features of compounds, providing them antioxidant activities, are closely connected with their reactivity with molecules that should be in the same time protected (proteins).

4. Conclusions

The influences of antioxidants of natural origin on the catalytic activity of AST and on the enzyme glycation by fructose were evaluated using two different enzyme assays. Both beneficial and possible undesirable effects of antioxidants were found. The authors believed that these equivocal effects should be considered in view of various conditions (e.g. concentration, presence of other compounds) before the use of the compounds is generally recommended. The compounds with beneficial effect may join the list of antiglycation compounds described so far.

Acknowledgements

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Glycation-induced inactivation of aspartate aminotransferase, effect of uric acid

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Abstract

Glycation is common posttranslational modification of proteins impairing their function, which occurs during diabetes mellitus and aging. Beside extracellular glycation of long-lived proteins, intracellular modifications of short-lived proteins by more reactive sugars like fructose are possible. The process includes free oxygen radicals (glycoxidation). In an attempt to reduce glycoxidation and formation of advanced glycation products (AGE), influence of 0.2–1.2 mM uric acid as endogenous antioxidant on glycoxidation of purified pig heart aspartate aminotransferase (AST) by 50 mM and 500 mM D-fructose *in vitro* was studied. Uric acid at 1.2 mM concentration reduced AST activity decrease and formation of total AGE products caused by incubation *in vitro* of the enzyme with sugar up to 25 days at 37 °C. The results thus support the hypothesis that uric acid has beneficial effects in controlling protein glycoxidation. The *in vitro* system AST-fructose proved to be a useful tool for investigation of glycation process. (Mol Cell Biochem **278:** 85–92, 2005)

Key words: aspartate aminotransferase, endogenous antioxidant, glycoxidation, non-enzymatic glycation, uric acid

Introduction

Proteins in the body are continuously modified by glycation and oxidation (glycoxidation) reactions, also known as Maillard reactions. Schiff base and Amadori adducts between free amino groups of proteins and carbohydrate-derived carbonyls eventually give rise to a broad range of advanced glycation end products (AGEs). Long-lived proteins, such as extracellular collagen, accumulate significant amounts of AGEs overtime [1]; however, even short-lived proteins, such as serum [2] and intracellular proteins, accumulate amounts of AGEs. Some data have shown that AGEs levels increase much more rapidly inside cells than they do on the extracellular matrix when cells were incubated in high glucose media [3]. This suggests that hyperglycemia causes rapid changes in intracellular sugars that are more reactive glycating agent than glucose, such as fructose as a result of sorbitol pathway activation, in addition to glycolysis [4].

The increased protein glycation and accumulation in tissue proteins are hallmarks of chemical consequence of chronic hyperglycemia, involved in damaging tissue proteins through alterations of their structure and function and are thought to be involved in the pathogenesis of diabetic complications [5]. Important components of this process are free oxygen radicals and more or less toxic intermediate and advanced glycation products (AGEs). Due to their generation, the process may be called glycoxidation. Some AGEs have typical

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fluorescent properties, which can be used for their detection and quantification [6].

Attempts have been made to reduce glycation process by antioxidants. *In vitro* studies showed that sulfhydryl compounds and antioxidants can significantly inhibit AGEs formation [7], presumably by reducing transition metal catalysis of oxygen radical reactions and by scavenging various free radical species [8, 9].

Uric acid is an endogenous compound traditionally considered to be a metabolically inert end-product of purine metabolism in man, without any physiological value. However, this ubiquitous compound has proven to be a selective antioxidant, capable especially of reaction with hydroxyl radicals and hypochlorous acid, itself being converted to innocuous products (allantoin, allantoate, glyoxylate, urea, and oxalate) [10]. Moreover, uric acid is able to chelate iron ions and thus inhibits iron-catalyzed oxidation of biomolecules [11].

As far as we know, there are no data in literature that would describe an influence of uric acid on the process of nonenzymatic glycation, especially AGEs. Therefore, the present paper aims to investigate possibilities of intervention of uric acid to an advanced glycation process.

Materials and methods

Chemicals

Aspartate aminotransferase: Purified enzyme preparations (291 U/mg protein and 25 mg, cytosolic enzyme from the porcine heart, suspended in saturated ammonium sulfate) from Serva Electrophoresis GmbH, Germany, and Roche Diagnostics, Mannheim, Germany, were used. D-Fructose was obtained from Sigma Chemical Co., USA. Uric acid and sodium azide were purchased from Lachema, Brno, Czech Republic. All chemicals used were of analytical grade.

Sample preparation and incubation

The suspension of aminotransferase was centrifuged at 5000 rpm at 4 °C for 20 min. The supernatant was removed and the enzyme pellet dissolved in an appropriate amount of 0.1 mol/l sodium phosphate buffer (pH 7.4) with 0.05% sodium azide to yield an enzyme stock solution with a concentration of 2.66 mg/ml. Following this, the enzyme stock solution was used to prepare six types of incubation samples: (a) with buffer only (control samples); (b) with uric acid in a final concentration of 0.2, 0.4, 0.6, or 1.2 mM; (c) with D-fructose in a final concentration of 50 mM; (d) with D-fructose in a final concentration of 500 mM; (e) with D-fructose in a final concentration of 50 mM and uric acid in the range of concentrations mentioned above; (f) with D-fructose in a final concentration of 500 mM and uric acid. The final concentration of the enzyme was 1.33 mg/ml. The enzyme concentration used was based on our previous experience [12]. Samples were incubated up to 25 days at 37 °C.

Determination of aspartate aminotransferase activity

Sample aliquots were removed at time zero and on 1, 5, 8, 12, 15, and 21 days of incubation. Aliquots were diluted 2000-fold with 0.1 mol/l phosphate buffer, pH 7.4, and AST activity was assayed in triplicates on a Hitachi 917 analyzer (Roche Diagnostics, Mannheim, Germany) using the common kinetic UV-method with addition of pyridoxal-5′-phosphate to the incubation mixture. Samples were diluted to give catalytic activity approximately about 6 μkat/l, which made it possible to follow activity changes during experiments using the common method of clinical laboratory. The intervals for the AST assay were chosen in order to cover the time-course of enzyme activity development during the incubation, as well as to exploit the capacities of the clinical laboratory. AST activity of Precinorm U and Precipath U (all from Roche Diagnostics, Mannheim, Germany) was measured before and after each assay. The between-run coefficients of variation of the assay for Precinorm U and Precipath U were lower than 2.0%. All results of enzyme assays were expressed in μkat/l and usually recalculated as activities relative to those of the respective control samples.

Determination of fluorescent AGE products

Sample aliquots (200 μl) were removed at time zero and on days 1, 3, 5, and 25 of incubation. Unlike AST activity decrease, the fluorescence development was less rapid and that was why a lower number of intervals were chosen. After collection, the aliquots were frozen and stored at −80 °C. After defrosting, the samples were put on the well-plate and the fluorescence at specific wavelengths ($\lambda_{ex}/\lambda_{em}$) corresponding to total AGEs (370/440 nm) and to pentosidine (335/385 nm) was measured. Fluorescence of samples was assayed on a luminescence spectrometer Perkin-Elmer LS 50B. Results were expressed in AU per mg of protein. One AU corresponded to the fluorescence of bovine serum albumin at a final concentration of 1.0 mg/ml.

Determination of pI of glycated aspartate aminotransferase

The isoelectric focusing was performed to determine the effect of glycation on pI of the enzyme. Sample aliquots (30 μg protein) were precipitated by ethanol and resuspended in rehydratation buffer (9 M urea, 2% CHAPS, 2% Pharmalytes,

20 mM DTT, 0.0025% blue bromophenol). The ImmobilineDrystrips (pH 3–10, 18 cm) were allowed to rehydrate overnight and electrofocusing carried out in a Multiphor II device (Amersham Biosciences) for 2145 Vh. The gels were then stained with 1% Coomassie blue R250, dried, scanned, and pI was calculated from R_f using the Imagemaster 2D Elite software (Amersham Biosciences).

Effect of glycation on molecular charge of the aspartate aminotransferase

Native polyacrylamide gel electrophoresis (native PAGE) was used to investigate the change in the molecular charge of AST due to glycation. Electrophoresis was performed on a 7.5% polyacrylamide non-denaturing gel. Protein (15 μg) was dissolved in sample buffer without denaturing (SDS) and reducing (DTT) agents using a minicell (Mini-ProteanII: Bio-Rad), and electrophoresis was performed at 30 mA per gel for 2 h. The gel was stained and scanned, and relative migration distances were calculated from R_f using the Imagemaster software (Amersham Biosciences). Electrophoretic mobility was expressed as a rise in percentage mobility compared to the native enzyme (control).

Statistical analysis

Values of catalytic activity are given as means ± S.D. and mostly expressed in % of control samples ± relative S.D. Values of fluorescence (AU/mg protein) are given as means ± S.D. Student's *t*-test is used to compare data. A *p*-value of <0.05 is considered as statistically significant.

Results

AST showed high stability during experiments [12], which can be demonstrated by catalytic activities of control samples. Some day-to-day fluctuations were caused mostly by daily setup of the assay in the laboratory. Therefore, the results were also recalculated in relation to daily controls (AST only) (see Fig. 1). The results showed the influence of D-fructose (Frc) on AST with a gradual time-dependent and sugar concentration-dependent decrease in enzyme activity under the influence of D-fructose. The effect on AST of both 50 mM and 500 mM D-fructose was progressive, with a rapid decrease in enzyme activity starting on the 1st day. Decrease in enzyme activity caused by D-fructose 50 mM was about 35% and by D-fructose 500 mM 46% after 5 days of incubation. Differences between the activities of AST versus AST + Frc 50 mM and AST versus AST + Frc 500 mM are statistically significant since the 5th and 1st day ($p < 0.05$ and $p < 0.01$), respectively. Figure 1 also demonstrates that uric acid itself had no direct harmful effect on enzyme stability and enzyme activity in the concentrations used. On the other hand, the compound in combination with D-fructose 50 mM showed a positive antiglycating effect, which was statistically significant on days 8, 12, 15 ($p < 0.05$) or 8, 15 ($p < 0.01$). A positive effect of uric acid in the presence of fructose 500 mM was less remarkable and it was significant on days 1, 12, and 15 ($p < 0.05$ and $p < 0.01$).

Figure 2 shows formation of "non-specific" (total) fluorescent AGE products in the presence of fructose. For the sake of clarity, only intervals 0, 5, and 25 days of incubation are presented in the Fig. 2. Fluorescence of AST (control sample) and AST + uric acid (1.2 mM) did not vary in the course of experiment (an average was 1.55 ± 0.05 AU/mg for AST and 1.51 ± 0.04 AU/mg for AST + UA). The increase in the fluorescence signal in the presence of fructose in both concentrations is obvious and obtained data are significant since the 3rd day for fructose 500 mM (2.65 ± 0.12 AU/mg) and since the 5th day for fructose 50 mM (2.19 ± 0.57 AU/mg), respectively. Fructose in higher concentration had stronger effect on glycation of AST. Uric acid showed a positive antioxidative (antiglycating) effect, which was statistically significant only on the day 5 ($p < 0.05$) in the presence of fructose 500 mM.

Formation of pentosidine in the presence of fructose is demonstrated in Fig. 3. Fluorescence of AST (control sample) and AST + uric acid (1.2 mM) did not vary in the course of experiment (an average was 2.30 ± 0.28 AU/mg for AST, and 2.27 ± 0.23 AU/mg for AST + UA). Increase in fluorescence in the presence of fructose in both concentrations is obvious and obtained data are significant on the 25th day for fructose 500 mM (7.66 ± 0.72 AU/mg) and on the 3rd and 25th day for fructose 50 mM (2.99 ± 0.75 AU/mg and 6.44 ± 2.05 AU/mg), respectively. Fructose in higher concentration had stronger effect on glycation of AST. In conclusion, uric acid showed a weak inhibitory effect on the formation of fluorescent products.

Effect of glycation on molecular charge of the enzyme

The appearance of anionic structure due to glycation was analyzed by electrophoretic mobility in native PAGE electrophoresis (Table 1). Compared to the mobility of the native enzyme lane 0, the enzyme incubated with 500 mM D-fructose alone (line AST + Frc) exhibited a significant increase in mobility up to 8.7% on the 5th day of incubation, and reached 20.9% on the 25th day. The enzyme incubated in the presence of both D-fructose and uric acid (line AST + Frc + UA) exhibited a smaller rise in mobility, up to 5.8% within 5 days and to 9.3% on the 25th day. These data indicated that the enzyme molecule became more anionic due to glycation and that uric acid had a light but significant inhibitory effect on the late glycation process.

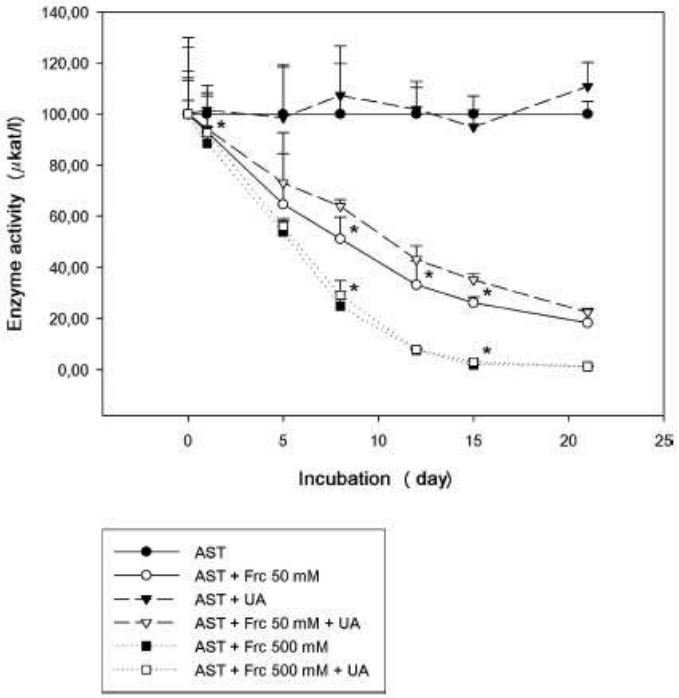


Fig. 1. Effect of glycation on AST activity and its intervention by uric acid. AST was incubated with or without fructose (50 mM and 500 mM) in 0.1 mM phosphate buffer, pH 7.4 at 37 °C in the presence or absence of uric acid (1.2 mM). Activity was expressed as percentage of activity of the control sample (without sugar), which was 100% at every interval ± S.D. (%). Every point in 0th–12th day represents an average of three experiments and on the 15th and 21st day an average of two experiments, in which assays were performed in triplicates (*data with $p < 0.05$, Student's *t*-test).

Table 1. Effect of glycation on molecular charge of aspartate aminotransferase

Incubation (days)	1	3	5	25
E + F	0	5.80	8.70	20.93
E + F + UA	1.45	4.35	5.80	9.30

The electrophoretic mobility of the enzyme on native PAGE electrophoresis was expressed as increased percentage compared to the control (100%). E = aspartate aminotransferase, F = D-fructose 500 mM; UA = uric acid 1.2 mM.

Determination of pI of glycated enzyme

We used the isoelectric focusing technique to follow the shift in pI of the enzyme as an index of glycation since glycation should lead to a more anionic molecule. Four bands were observed in the control sample corresponding to pI 7.40, 7.13, 6.92, and 6.80 (Table 2), indicating that sample enzyme was constituted of at least four isoforms, since the theoretical value for AST pI is 6.80 (lane 1). When incubated with 500 mM D-fructose (line 1), the 3rd and the 4th bands shifted towards acidic pH within 5 days of incubation to 6.85 and 6.64, respectively, while the 1st fraction (pI 7.4) underwent an important shift to pI 6.5 only after 25 days (lane 2). Incubated in the presence of both fructose and uric acid (line 2), the four bands did not move up to the 5th day of incubation, when after 25 days we observed two new bands with pI of 6.50 and 6.30. These data indicated that the enzyme underwent glycation when incubated with D-fructose leading to a shift down of pI and that uric acid had a protective effect against late glycation, corroborating the above results.

Discussion

The *in vitro* model used in this study consists of crucial molecules (protein-sugar-potential antiglycating compound) that would interact similarly in the process of glycation under conditions *in vivo*. We tried to simplify the model as much as possible in order to have one protein in the system, which

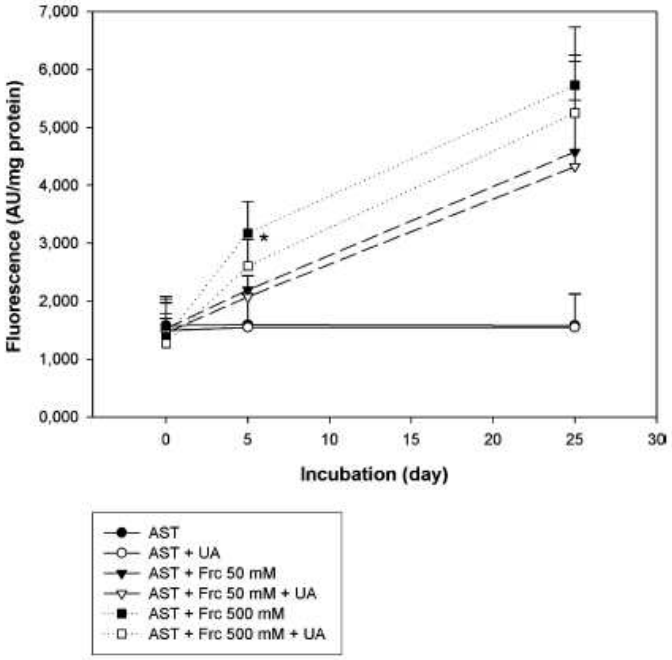


Fig. 2. Formation of fluorescent products of glycation under the conditions of long lasting incubation. AST was incubated with or without fructose (50 mM and 500 mM) in 0.1 mM phosphate buffer, pH 7.4 at 37 °C in the presence or absence of uric acid (1.2 mM) up to 25 days. Aliquots of samples were taken on days 0, 1, 3, 5, 25, and arising fluorescent AGE products in samples were determined at specific wavelengths of excitation and emission ($\lambda_{ex}/\lambda_{em}$) corresponding to Total AGEs (370/440 nm). Data of relative fluorescence were expressed in arbitrary units per mg of protein ± S.D., with 1AU corresponding to the fluorescence of BSA 1.0 mg/ml. Every point in days 0 and 5 represents an average of four experiments (10 samples), in days 1 and 3 an average of three experiments for mixtures with fructose 50 mM (7 samples) and of two experiments for mixtures with fructose 500 mM (4 samples), and in day 25 an average of three experiments (7 samples), (*data with $p < 0.05$, Student's *t*-test).

would be representative enough for a mixture of cellular proteins and which change under glycation could be simply measured [12]. The pig heart aspartate aminotransferase (AST, EC 2.6.1.1) as the model protein was used, which was incubated with D-fructose *in vitro* up to 25 days at 37 °C. This enzyme plays a fundamental biochemical role in the metabolism of amino acids and it is used as a marker of cell damage in clinical chemistry. Liver and kidney cAST activity decreases in diabetes [13] suggesting that this enzyme is modifiable. Regardless of its metabolic and diagnostic role, the enzyme represents a good model for protein glycation studies. It is very stable *in vitro* for a long time of incubation. Aspartate aminotransferase contains several lysine residues in the molecule and has been remarkably affected by fructose during glycation process. The process of losing the catalytic function of AST can be easily monitored [12]. The enzyme is available in a purified form, permitting thus to characterize intramolecular changes of the protein during glycation. D-fructose was used because this sugar proved to be a more efficient glycating agent of AST than glucose according to previous experiments [12, 14], while the principle of the reaction chain is the same. Since protein glycation by fructose (fructation) has been shown to generate fluorescent products at a high rate [15], fluorescence of AGEs is used to investigate AGEs production during the process. The method turned out well in a study of AST glycation by glyceraldehyde phosphate [16].

Comparing the *in vitro* conditions in the model with those *in vivo*, it may be admitted that concentrations of individual components of the *in vitro* system are higher than under physiological or pathological conditions *in vivo*. This is especially the case of AST preparation as a model protein, which stability during the experiment was crucial, and the preparation was very unstable at low concentrations (preliminary results during preparation of the model, not published). Concentration of glycating sugar was then partly adapted to the high

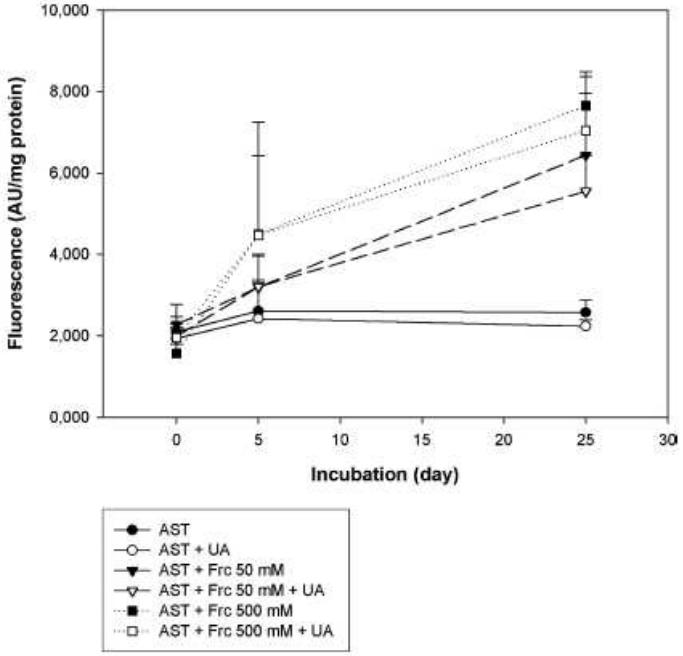


Fig. 3. Formation of pentosidine under the conditions of long lasting incubation. AST was incubated with or without fructose (50 mM and 500 mM) in 0.1 mM phosphate buffer, pH 7.4 at 37 °C in the presence or absence of uric acid (1.2 mM) up to 25 days. Aliquots of samples were taken on days 0, 1, 3, 5, 25 and arising fluorescent AGE products in samples were determined at specific wavelengths of excitation and emission ($\lambda_{ex}/\lambda_{em}$) corresponding to pentosidine (335/385 nm). Data of relative fluorescence were expressed in arbitrary units per mg of protein ± S.D., with 1AU corresponding to the fluorescence of BSA 1.0 mg/ml. Every point in days 0 and 5 represents an average of three experiments (9 samples), in days 1 and 3 an average of two experiments for mixtures with fructose 50 mM (6 samples) and of one experiments for mixtures with fructose 500 mM (3 samples), and in day 25 an average of two experiments (6 samples), (*data with $p < 0.05$, Student's *t*-test).

concentration of protein to bring the stoichiometry of reaction closer to conditions *in vivo*. As for uric acid, the two lower concentrations are within those *in vivo* under physiological conditions, while the 0.6 mM and 1.2 mM concentrations represent hyperuricaemia *in vivo*, which is the situation under which the compound should demonstrate its antioxidant and antiglycating effects.

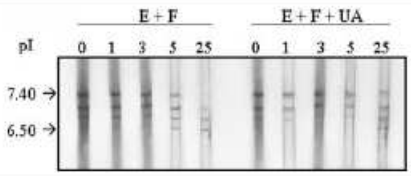
As for the AST assay, despite a daily setup of the assay in the clinical laboratory, some day-to-day fluctuations were noticed. These fluctuations come from the calibration procedures inside the laboratory. Their effect on results could have been overcome, when the results were related to the control activity of the day.

As for uric acid, different concentrations of the compound were investigated in preliminary experiments. The results suggested that 0.2 to 0.6 mM concentrations had neither direct nor indirect effect on AST and its glycation, respectively. Recent findings [17] demonstrate not only antioxidant but also pro-oxidant effects of uric acid and possible direct casual role of the compound in the pathogenesis of hypertension and atherosclerosis. Our results do not confirm such ambiguous role of uric acid. Nevertheless, the findings of equivocal role of uric acid in pathogenesis of these diseases and connection of hyperuricaemia with gout does not support any prospects of treatment of diabetes by uric acid.

The fact that 1.2 mM uric acid inhibits the decrease in AST activity caused by D-fructose documents a beneficial effect of the compound against glycation process under conditions comparable to hyperuricaemia *in vivo*. The effect is more remarkable in the presence of 50 mM D-fructose, while it seems to be less certain in the presence of 500 mM D-fructose. Virtually less remarkable beneficial intervention of uric acid in AST glycation by 500 mM D-fructose suggests that remaining AST activity is too low under the influence of such a high concentration of the reactive sugar and any beneficial influence of uric acid on this parameter is difficult to recognize. This consideration is supported by fluorescence measurements, the results of which show clearly an inhibiting

Table 2. Determination of pI of glycated aspartate aminotransferase

Incubation (days)	0	1	3	5	25
E + F	7.40	7.40	7.40	7.40	–
	7.13	7.13	7.13	7.13	7.13
	6.92	6.92	6.92	6.85	6.85
	6.80	6.70	6.70	6.70	6.64
					6.50
E + F + UA	7.40	7.40	7.40	7.40	7.40
	7.13	7.13	7.20	7.20	7.13
	6.85	6.85	6.92	6.92	6.85
	6.64				6.64
					6.50
					6.30



The enzyme was incubated with fructose alone (E + F) or in the presence of uric acid (E + F + UA) at different times and samples submitted to isoelectric focusing on pH 3-10 immobiline. E = aspartate aminotransferase; F = D-fructose 50 mM; UA = uric acid 1.2 mM.

effect of uric acid on the formation of AGE products even at higher sugar concentration. It was not possible to investigate higher concentration of uric acid for compensation of high concentration of sugar because of limited solubility of uric acid in water solutions.

As for fluorescence measurements, control samples as well as samples containing AST and with only uric acid showed stable fluorescence since the start of the experiment. Fluorescence of AST even at the start of the experiment was not negligible. Nevertheless, it should be taken into account that starting fluorescence of AST (representing 1.33 mg of the enzyme preparation) is comparable with that of BSA (representing 1 mg of protein). It seems that most of this fluorescence constitutes general fluorescence properties of proteins. The presence of pyridoxal-5′-phosphate coenzyme in the AST molecule may also contribute to basal fluorescence of the enzyme.

As for the sources of fluorescence in the presence of fructose, there are three routes to produce AGEs: that which occurs via the autooxidative pathway (sugar gives reactive products by autooxidation) [18], another one that follows the conventional Amadori rearrangement (AP is oxidized) [19], and that proceeding from the Schiff base [20]. All these ways may contribute to AGE formation in experiments with AST protein.

The fact that the uric acid inhibitory effect on glycation led to a lower level of fluorescence linked to AGE adducts suggests that uric acid acts at least in part by trapping dicarbonyl intermediates in AGE formation, the potential mechanism requiring further investigation to be elucidated.

The physicochemical analysis (IEF and native electrophoresis) confirmed the results obtained by other methods, i.e., changes in the protein molecule in the presence of fructose and inhibition of the glycation process by means of uric acid. The glycation-induced chemical modifications led to a change in molecular charge of the enzyme, which became more anionic and consequently pI shifted down to acidic pH. We attempted to delay the glycation process using uric acid as a potential inhibitor of advanced glycation end products formation.

In conclusion, inhibitory effect of uric 1.2 mM acid against formation of total AGE formation seems to be remarkable. These results thus contribute to investigations supporting the hypothesis that uric acid belongs to compounds that may have beneficial effects [10] in the organism, by inhibition of deterioration, represented in this case by glycation of proteins under conditions comparable to hyperuricaemia *in vivo*. Nevertheless, we have not found in literature any evidence of connection of hyperuricaemia with lower levels of AGE in such patients.

There is evidence that liver and kidney cAST activity decreases in diabetes suggesting that this enzyme is modifiable [13]. Nevertheless we stress that the used system AST-fructose represents first of all a simple useful model protein for *in vitro* glycation study and no metabolic consequences *in vivo* should be deduced from the results.

Acknowledgments

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Oxidative stress parameters in different systemic rheumatic diseases

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Abstract

The involvement of oxidative stress in the pathogenesis of rheumatic disorders, such as systemic sclerosis (SSc) and chronic polyarthritides, has been suggested yet not thoroughly verified experimentally. We analysed 4 plasmatic parameters of oxidative stress in patients with SSc (n = 17), psoriatic arthritis (PsA) (n = 10) and rheumatoid arthritis (RA) (n = 9) compared with healthy subjects (n = 22). The biomarkers were: total antioxidant capacity (TAC) measured by ferric reducing antioxidant power (FRAP) method, hydroperoxides determined by ferrous ion oxidation in presence of xylenol orange (FOX) method and sulfhydryl and carbonyl groups assessed by spectrophotometric assays. The results showed significantly increased hydroperoxides in SSc, PsA and RA (3.97 ± 2.25, 4.87 ± 2.18 and $5.13 \pm 2.36\ \mu mol\ L^{-1}$, respectively) compared with the control group ($2.31 \pm 1.40\ \mu mol\ L^{-1}$; $P < 0.05$). Sulfhydryls were significantly lower in SSc ($0.466 \pm 0.081\ mmol\ L^{-1}$), PsA ($0.477 \pm 0.059\ mmol\ L^{-1}$) and RA ($0.439 \pm 0.065\ mmol\ L^{-1}$) compared with the control group ($0.547 \pm 0.066\ mmol\ L^{-1}$; $P < 0.05$). TAC in all three diseases showed no difference in comparison with controls. Carbonyls were significantly higher in RA than in the control group (32.1 ± 42 vs $2.21 \pm 1.0\ nmol\ (mg\ protein)^{-1}$; $P < 0.05$). The obtained data indicate augmented free radical-mediated injury in these rheumatic diseases and suggest a role for the use of antioxidants in prevention and treatment of these pathologies.

Introduction

The involvement of oxidative (oxidant) stress has been proposed in the destruction of cartilage and connective tissue occurring in rheumatic disorders, such as systemic sclerosis and chronic polyarthritis. Systemic sclerosis is a generalized multisystemic disorder characterized by microvascular damage eventually leading to tissue fibrosis accompanied by a range of severe complications. Its aetiology and pathogenesis has not been fully understood, yet generally it is believed that the causes are rather complex. The vascular disease has been the pivotal aspect under investigation (Kahaleh 2004). There are many factors contributing to the inflammation and derangement of the vascular endothelium, mainly by alteration of immune processes or changes in the coagulative/fibrinolytic system (Simonini et al 1999). Oxidative stress has been proposed as one of the possible factors (Murrell 1993). The way the reactive oxygen and nitrogen species (RONS) can contribute to the pathogenesis of vascular disease is very extensive. They may damage endothelial cells' function directly by chemical modification of macromolecules via peroxidation of lipids and oxidation of proteins or by activating various pro-inflammatory cytokines, which further initiate cascades of processes leading to activation of fibroblasts and immune cells (Herrick & Matucci Cerinic 2001). Furthermore, RONS may well directly alter DNA and proteins from apoptotic cells and thus contribute to the development of autoimmune responses (Ahsan et al 2003).

Raynaud's phenomenon, present in most patients, is very often the first symptom to occur, usually preceeding the development of systemic sclerosis by months or even years. Raynaud's phenomenon provokes frequent episodes of hypoxia-reperfusion, thus producing RONS responsible for endothelial injury. However, Raynaud's

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phenomenon and related events are not the only possible source of free radicals in the microvasculature. Other factors include activated polymorphonuclear cells, which can produce high amounts of RONS along with proteases (Lau et al 1992a, b), and also inducible nitric oxide (NO) synthase, which can generate pathological excess of NO (Matucci Cerinic & Kahaleh 2002). These early events are often followed by small vessel structural changes and ischaemia. The birth of a vicious circle of RONS generation along with related inflammatory processes leads to further endothelial damage, obliteration of microvasculature and fibrosis (Murrell 1993; Kahaleh 2004). Yet since only a small percentage of patients with primary Raynaud's phenomenon eventually develop systemic sclerosis, there must be, besides increased RONS formation, apparently some concurrent factors present. It is probably an abnormal susceptibility to oxidative damage mostly due to deteriorated antioxidant defence system.

The involvement of oxidative stress in the pathogenesis of chronic polyarthritis, such as rheumatoid arthritis and psoriatic arthritis, has also been proposed (Halliwell 1995). Epidemiological studies have shown that rheumatoid arthritis occurs in previously healthy subjects with low levels of circulating antioxidants and, once established, is characterized by reactive oxygen products within affected joints. Besides, the local production of hydroxyl radicals seems to modify the structure of human IgG, thereby increasing the production of rheumatoid factors, the biological marker of rheumatoid arthritis. Along with ischaemia-reperfusion injury during joint movement, the affection of inflamed synovium by RONS is attributed mainly to activated neutrophils. These are, like in systemic sclerosis, kept at inflammatory sites by repeated formation of various chemotactic molecules, including cytokines, adhesive molecules, etc. Consequently, generated RONS cannot be effectively scavenged due to alterations in physiological antioxidant defences (Halliwell 1995; Taysi et al 2002).

The extent of free-radical-mediated injury is reflected in increased levels of different products of oxidative reactions. Since RONS can attack all biological macromolecules, there has been a wide range of assays determining various oxidative stress parameters in-vivo. The first sensitive targets for attacks of RONS are unsaturated fatty acids of cells' lipid membranes. This process yields many products, including hydroperoxides, conjugated dienes, aldehydes, F_2-isoprostanes, etc. Free-radical-mediated oxidation of proteins may similarly lead to many end-products.

The role of free radicals has been extensively studied in the pathogeneses of some diseases but less is known about their role in rheumatic diseases. The objective of this study was to assess four different parameters reflecting oxidative stress status changes in plasma and compare them with healthy controls and thus help to clarify the hypothesis of oxidative stress involvement in the pathogenesis of systemic sclerosis, rheumatoid arthritis and psoriatic arthritis.

Materials and Methods

Patients

Thirty-six patients with rheumatic disorders were studied, from which 17 suffered from systemic sclerosis, 9 from rheumatoid arthritis and 10 from psoriatic arthritis. All patients were recruited from the Department of Rheumatology, University of Rome "La Sapienza", in agreement with the Declaration of Helsinki (http://www.wma.net/e/policy/b3.htm). Systemic sclerosis and rheumatoid arthritis patients were diagnosed and classified according to the criteria of the American College of Rheumatology (Anon 1980; Arnett et al 1988); psoriatic arthritis was diagnosed according to the criteria proposed by Moll & Wright (1973). Systemic sclerosis patients were divided into cutaneous limited (10 cases) and diffuse (7 cases) subsets (LeRoy et al 1988). Patients were receiving various medications, most commonly corticosteroids, vasodilators and non-steroidal anti-inflammatory drugs. Where suitable, individual patients received also ciclosporin, azathioprine, methotrexate or cyclophosphamide. Eight patients with lower disease activity took no medication at all. In both inflammatory arthritides the most commonly taken drugs were methotrexate and low-dose corticosteroids; a few patients also took infliximab, leflunomide or hydroxychloroquine. A summary of the patients' main characteristics is shown in Table 1. The control group consisted of 22 healthy subjects with no acute or chronic inflammatory disease and on no medication, excluding also any antioxidant supplementation (9 males, 13 females; age 25–72, mean 40).

Table 1 Main demographic, clinical and laboratory parametres of the patients with systemic sclerosis (SSc), rheumatoid artritis (RA) and psoriatic arthritis (PsA)

Characteristic	Ssc (n = 17)	RA (n=9)	PsA (n = 10)
Age (years), mean (range)	60 (41–79)	58 (45–76)	57 (36–74)
Sex (female/male)	13/4	9/0	2/8
Disease duration (years), mean (range)	9.8 (1–39)	10.9 (2–38)	12.3 (1–28)
Erythrocyte sedimentation rate ($mm\,h^{-1}$), mean (range)	18.2 (4–62)	21 (10–35)	31.8 (5–86)
C-reactive protein ($mg\,L^{-1}$), mean (range)	*	12 (2–24)	30 (3–110)

*Only in 4 cases mildly (max 2-fold higher than physiological levels) increased.

Samples

Venous blood was collected into EDTA-coated tubes (for the measurement of carbonyl and sulfhydryl groups) or sodium citrate-coated tubes (for hydroperoxides (ROOHs) and total antioxidant capacity (TAC) measurements). Plasma was separated within 30 min by centrifugation at 1500 *g* for 10 min at 4°C. Fresh samples were analysed within 3 h of sample collection. The rest of the plasma samples were divided into portions and stored at −70°C within 1 h of sample collection for later measurements. ROOHs, TAC and sulfhydryl groups of plasma samples were measured in fresh samples. Carbonyl groups were assessed on the next day.

Total antioxidant activity of plasma

The FRAP assay (ferric reducing antioxidant power) was performed according to the method of Benzie & Strain (1996) with minor modifications. Briefly, to prepare the FRAP solution, 10 mL of acetate buffer 300 mM, adjusted to pH 3.6 with acetic acid, were mixed with 1 mL of ferric chloride hexahydrate 20 mM dissolved in distilled water and 1 mL of 2,4,6-tris(2-pyridyl)-s-triazine 10 mM dissolved in HCl 40 mM. Ten microlitres of plasma were added to 1.8 mL of a freshly prepared FRAP solution in borosilicate test tubes in quadruplicate and the absorbance was measured at 593 nm after 6 min of incubation at room temperature against a blank of acetate buffer. Each day of experiment, Trolox in two different concentrations (0.2 and 0.4 mM) was used to obtain a new calibration curve. TAC, expressed as Trolox equivalent concentration, was then calculated dividing the absorbance change (A6 − A0) by the slope of the calibration curve. A0 was considered the sum of the absorbance of plasma diluted 180 times and the absorbance of the FRAP solution.

Hydroperoxides

ROOHs were determined by the FOX assay (ferrous ion oxidation in presence of xylenol orange) (Nourooz-Zadeh 1999) with minor modifications. All glassware was cleaned with warm concentrated nitric acid before use. Briefly, FOX solution was a mixture of two solutions, A and B. Solution A was prepared by dissolving butylated hydroxytoluene in pure methanol at 4.4 mM concentration. Solution B consisted of xylenol orange 1 mM and ammonium ferrous sulfate 2.5 mM dissolved in sulfuric acid 250 mM. Working solution was prepared by mixing A and B solutions at a proportion of 9:1, respectively. Working solution was kept at 4°C for a maximum of two weeks. The molar extinction coefficient for each freshly prepared working solution was determined by a calibration curve with different concentrations of hydrogen peroxide in the range 0.1–2.5 μM. The concentration of hydrogen peroxide was measured spectrophotometrically ($\varepsilon_{240} = 43.6\ cm^{-1}\ M^{-1}$) (Nourooz-Zadeh et al 1994). For measuring ROOHs in plasma, 90 μL of plasma was mixed with 10 μL triphenylphosphine 20 mM in methanol (in quadruplicate) or with 10 μL of methanol (in quadruplicate) in 1.5 mL microcentrifuge vials. The vials were vortex-mixed for 10 min each and incubated at room temperature in the dark for 30 min before adding 900 μL of FOX solution. Then the samples were again incubated for 1 h at room temperature in the dark, being vortex-mixed each for 10 min, and centrifuged at 16 000 *g* for 7 min. The absorbance of the supernatant was determined at 560 nm by spectrophotometer (Hewlett-Packard 8452AX). The absorbance of the samples treated with triphenylphosphine was subtracted from non-treated samples to calculate the concentration of ROOHs.

Sulfhydryl groups

Sulfhydryl groups/thiols in plasma were measured by a spectrophotometric method using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent) (Hu 1994) with minor changes. Briefly, 60 μL of plasma were mixed with 200 μL of Tris-EDTA buffer (Tris base 0.25 M, EDTA 20 mM, pH 8.2) in 1.5-mL microcentrifuge tubes in quadruplicate. Subsequently, 15 μL of DTNB 10 mM in methanol were added to the samples, except one blank to which the same quantity of methanol was added. After 15 min of incubation at room temperature in the dark, 1 mL of methanol was added to all samples and they were centrifuged at 3000 *g* for 10 min after 5 min of incubation. The absorbance of the supernatant was then measured at 412 nm on a spectrophotometer and the concentration of sulfhydryl groups was calculated ($\varepsilon = 13600\ cm^{-1}\ M^{-1}$).

Carbonyl groups

Carbonyl groups in plasma were determined using the reagent 2,4-dinitrophenyl hydrazine (DNPH) as described previously (Levine et al 1990; Firuzi et al 2003) with minor changes. Briefly, in 1.5-mL microcentrifuge tubes 15 μL of plasma were mixed with 400 μL of DNPH 15 mM in HCl 2 N in quadruplicate. Four-hundred microlitres of HCl 2 N were added to 15 μL of plasma in duplicate as blanks. The samples were vortex-mixed for 10 min each and after 1 h of incubation at room temperature in the dark, 1 mL of trichloroacetic acid 14% (w/v) was added. After another 10 min of incubation all the samples were centrifuged at 11 000 *g* for 5 min. Then the supernatant was discarded and precipitates were washed 3 times with 1 mL of a mixture of ethanol–ethylacetate (1:1, v/v) and, after 10 min, centrifuged. Finally, the protein precipitates were dissolved in 900 μL of guanidine hydrochloride 6 M in KH_2PO_4 20 mM adjusted to pH 2.3 with diluted trifluoroacetic acid. Subsequently, the samples were placed in a water-bath at 37°C for 1 h and further incubated for 2 h in the dark at room temperature. The absorbance was measured at 360 nm with a spectrophotometer against guanidine solution as a blank. The concentration of carbonyls was calculated using the molar extinction coefficient (ε) 22000 $cm^{-1}\ M^{-1}$. The protein concentration in the samples was determined by the modified Bradford assay (Macart & Gerbaut 1982). Measurements were performed by a

microplate reader (Bio-Rad 3550) at 595 nm and calibration curves were prepared with bovine serum albumin as a standard.

Statistics

The obtained data were analysed using one-way analysis of variance with Holm-Sidak (all versus control) post-hoc test performed with SigmaStat version 3.00 for Windows (SPSS Inc., Chicago, IL). The same software was used for Pearson's correlation analyses. $P < 0.05$ was considered statistically significant. All quantitative data are expressed as the mean ± standard deviation.

Results

Total antioxidant capacity

Raw data and means are shown in Figure 1A (expressed as molar concentration equivalent of Trolox, a water-soluble analogue of α-tocopherol). The total antioxidant capacity (TAC) was neither in systemic sclorisis nor in any arthritis significantly different from the control group. The values in patients tended to be even higher though the elevation was not significant. Furthermore, TAC in systemic sclerosis was found to negatively correlate with the disease duration ($P < 0.05$). The mean TAC value was 0.395 ± 0.095 in systemic sclerosis, 0.329 ± 0.074 in rheumatoid arthritis, 0.399 ± 0.142 in psoriatic arthritis and 0.327 ± 0.068 mmol L^{-1} (equivalent of molar concentration of Trolox) in the control group, respectively.

Hydroperoxides

The plasma levels of hydroperoxides (ROOHs) in all three groups are shown in Figure 1B. The mean values indicate that in all three diseases the ROOHs were increased compared with the control group ($P < 0.05$). Both inflammatory arthritides showed ROOHs even higher than systemic sclerosis. The mean level of hydroperoxides was 3.97 ± 2.24 in systemic sclerosis, 5.13 ± 2.36 in rheumatoid arthritis, 4.87 ± 2.18 in psoriatic arthritis and 2.31 ± 1.41 μmol L^{-1} in healthy controls, respectively.

Sulfhydryl groups

Plasma sulfhydryls were decreased, as compared with healthy controls, in all three groups of patients ($P < 0.05$). The scatter of sulfhydryl values, as well as the position of the mean, in all four groups are shown in Figure 2A. The lowest level was found in rheumatoid arthritis. In addition, in systemic sclerosis a significant negative correlation was found between elevated plasma hydroperoxides and decreased sulfhydryl groups ($P < 0.01$; Figure 3). In psoriatic arthritis patients sulfhydryls were negatively correlated with patients' ages ($P = 0.04$) and there was also a correlation with C-reactive protein levels ($P = 0.064$). The mean level of sulfhydryls was 0.466 ± 0.081 in systemic sclerosis, 0.439 ± 0.069 in rheumatoid arthritis, 0.467 ± 0.057 in psoriatic arthritis and 0.547 ± 0.066 mmol L^{-1} in the control group, respectively.

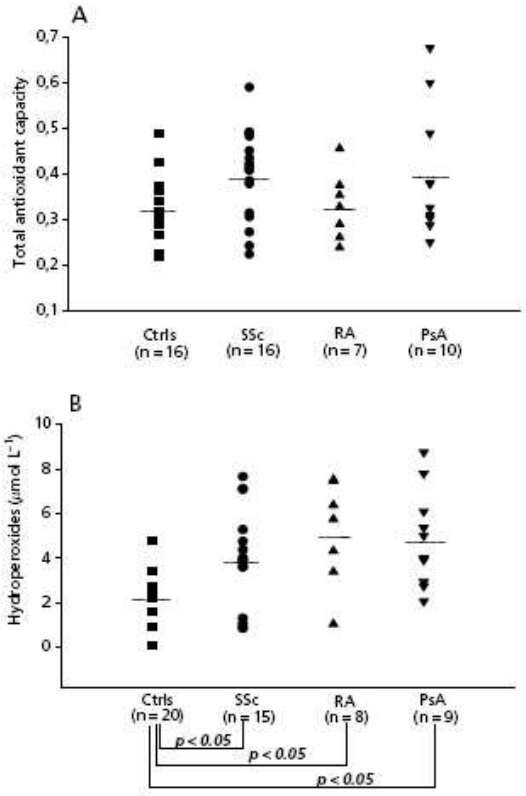

Figure 1 Total antioxidant capacity (A) and plasmatic hydroperoxides (B) in control group (Ctrls), systemic sclerosis (SSc), rheumatoid arthritis (RA) and psoriatic arthritis (PsA). The mean values for each group are shown as a horizontal line. Total antioxidant capacity is expressed as mmol L^{-1}, concentration equivalent to Trolox.

Carbonyl groups

The amount of carbonyl residues in systemic sclerosis did not differ from the control group. Neither did it in psoriatic arthritis (Figure 2B). In rheumatoid arthritis patients, however, two different groups were formed, one (24%, n = 3) having a level 70- to 90-fold higher than the rest. Carbonyl levels were thus on average more than 10-fold higher (32.08 ± 41.95 vs 2.21 ± 1.03 nmol (mg of protein)$^{-1}$) compared with the control group ($P < 0.001$). No difference in other markers or available clinical characteristics was found in the respective three patient groups, though. Mean values were 2.22 ± 0.84 in systemic sclerosis, 32.1 ± 41.94 in rheumatoid arthritis, 2.67 ± 2.05 in psoriatic arthritis and 2.21 ± 1.03 nmol (mg protein)$^{-1}$ in healthy controls.

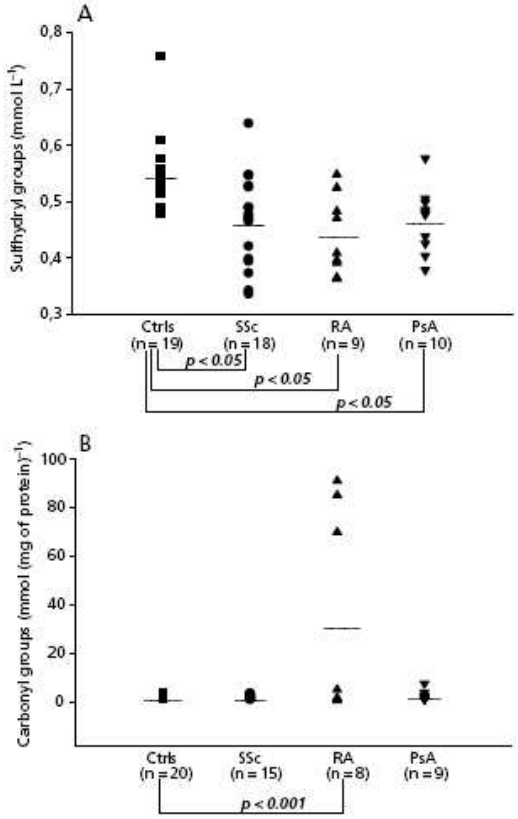

Figure 2 Sulfhydryl (A) and carbonyl (B) groups in control group (Ctrls), systemic sclerosis (SSc), rheumatoid arthritis (RA) and psoriatic arthritis (PsA). The mean values are shown as a horizontal line.

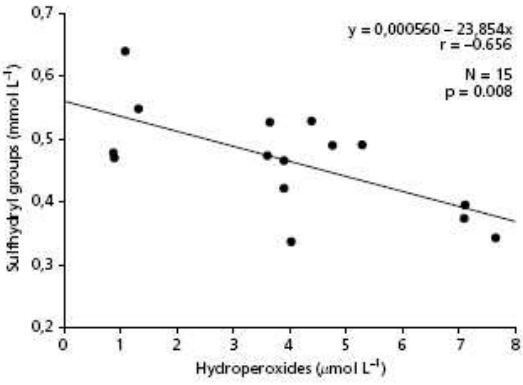

Figure 3 Linear regression between plasmatic hydroperoxides and sulfhydryl groups in systemic sclerosis.

Discussion

This study investigated and confirmed increased oxidative stress occurring in systemic sclerosis, rheumatoid arthritis and psoriatic arthritis. Under conditions of oxidative stress there is a host of various reactive species generated. These oxidants, mostly free radicals, are capable of attacking all biological macromolecules giving rise to different end-products. Therefore a battery of assays has been proposed for evaluation of oxidative stress in-vivo. The criteria for methods used in this study were for them to be fast and simple, thereby rendering them possible to be performed together soon after sample collection so as to prevent any further oxidative reactions. The assays we employed were FRAP (ferric reducing antioxidant power) (Benzie & Strain 1996), assessing total antioxidant capacity (TAC), FOX (ferrous ion oxidation in presence of xylenol orange) method (Nourooz-Zadeh et al 1994) measuring hydroperoxides (ROOHs), a marker of lipid peroxidation, and assays for determination of sulfhydryl (Hu 1994) and carbonyl groups (Levine et al 1990), both reflecting oxidation of proteins.

In systemic sclerosis, two of four investigated parameters were found to be significantly different from the control group – plasmatic ROOHs and sulfhydryl groups. Moreover, these markers clearly inversely correlate with each other, suggesting that free-radical-mediated injury occurs in systemic sclerosis. Elevated ROOHs also confirm previously reported increases in lipid peroxidation, although other studies evaluated different markers (Simonini et al 1999). The decrease in plasmatic thiols also indicates oxidation of proteins. Similar reduction of sulfhydryl groups, further associated with white blood cell activation, has been also been previously reported (Lau et al 1992a). However, this was not supported by the carbonyl-group-evaluating assay, where no significant difference was found. In contrast to our results, in the only previously made study evaluating carbonyl residues they were found higher than in control group (Borderie et al 2004).

Inconsistent with these findings that implicate an increase of oxidative stress in systemic sclerosis, TAC was not decreased as anticipated. Moreover, it was even higher than in healthy controls. This also goes against the assumption based on other authors' studies describing impairment in antioxidant defences in systemic sclerosis. Some of these studies reported decreased plasmatic levels of micronutrients participating in scavenging of free radicals, such as ascorbic acid, α-tocopherol, β-carotene or selenium (Lundberg et al 1992; Herrick et al 1994). Besides these studies investigating single blood levels of antioxidants, there are no previous reports on plasma antioxidant capacity evaluation by other authors. Even though the FRAP assay was originally designed for, and shown capable of, representing changes in antioxidant properties of biological fluids, it must be noted that the reaction conditions are far from physiological and in-vivo hierarchies and activity of individual antioxidants may not be directly related to (and thus represented by) the reducing

potential of such a complex sample as plasma. Therefore the test measuring reducing potential may lack enough sensitivity and fail to reveal minor changes in antioxidant defences. Another hypothetical explanation of the lack of significant changes in TAC may lie in some as yet unknown feedback mechanism being a response to increased oxidative stress (Prior & Cao 1999). Likewise adaptation to increased oxidative stress has been so far reported in the case of antioxidant enzymes and considering endogenous molecules, such as ceruloplasmin or glutathione, they could be overproduced to manage the rise of RONS. Even though none of these molecules could per se contribute to FRAP (or other TAC-measuring) assay, their antioxidant/chelating properties may in part replace other low molecular antioxidants (ascorbic acid, α-tocopherol, etc.) in binding free radicals and cause them to remain in their reduced forms, thus keeping the overall TAC higher. Indeed, accepting such an assumption, we could explain the inverse correlation between TAC and disease duration we found in systemic sclerosis (data not shown). Nonetheless, previously stated findings suggest that oxidative stress, according to the three of four markers evaluated, is more pronounced in the later stages of the disease. It includes oxidation of proteins (signified by reduced sulfhydryl groups), deterioration of antioxidant defences (decreased TAC) and also lipid peroxidation (elevated ROOHs). In a study evaluating lipid peroxidation, higher oxidative damage in the early stages of the disease was reported (Simonini et al 1999). This contrast may be attributed to different ways of assessing the progress of systemic sclerosis. It may be also due to the different accumulative properties of the markers under examination as well as to different molecular mechanisms leading to their formation. There was no difference in any of the four oxidative stress parameters found between limited and diffuse subsets of systemic sclerosis.

In rheumatoid and psoriatic arthritis, all four assays showed similar results to those of systemic sclerosis. The present data are among the first to be reported on oxidative stress status in psoriatic arthritis. In both arthritides, ROOHs were higher than in the control group. This difference was even greater than in systemic sclerosis. An array of studies on oxidative stress status in rheumatoid arthritis has been performed and some of them describe augmented lipid peroxidation in rheumatoid arthritis as well (Gambhir et al 1997; Taysi et al 2002). Even though different markers were determined, these reports confirm our findings. Plasma thiols were significantly lower in both diseases when compared with healthy controls; in rheumatoid arthritis they were the lowest of all the three diseases. This is in concordance with other authors' reports (Jaswal et al 2003). Further supporting the idea of increased oxidation of proteins in rheumatoid arthritis, we detected increased levels of carbonyl groups. Although higher amounts of carbonyl residues in rheumatoid arthritis have been previously reported by other authors (Mantle et al 1999; Renke et al 2000), the mean increase found in our study (13 fold) is remarkably high. Yet this statement needs to be regarded with particular caution, since high diversity was found among the patients, clearly separating them into two groups. No other difference (in other oxidative stress markers, biochemical data, type of medication, etc.) explaining this remarkable occurrence was observed, though (see Results). It would be certainly interesting to further extend the group of patients and investigate this issue more in detail. Nevertheless, the finding of very low sulfhydryl groups and elevated carbonyl groups indicates a serious oxidative injury, particularly in proteins. However, no correlation between these two parameters was observed. Unlike in the other diseases, sulfhydryl groups in psoriatic arthritis are inversely correlated with the age of patients. There is also an attenuated inverse correlation showing sulfhydryl groups decreasing as C-reactive protein levels increase, which may suggest an increase in the oxidation of proteins along with the severity of inflammation. TAC was not found in any arthritis to be significantly different from the control group. One study evaluating oxidative stress in rheumatoid arthritis (De Leo et al 2002) described a decreased TRAP (total radical trapping antioxidant parameter) value compared with controls. To explain our opposing results we can adopt the hypothesis suggested above for systemic sclerosis, that increased oxidative stress in-vivo may be faced by some inducible compensatory mechanisms other than only free-radical-scavenging enzymes. The results of studies evaluating blood antioxidants levels in RA (Gambhir et al 1997) have also proposed such an explanation.

In conclusion, our data support the hypothesis that oxidative stress occurs in systemic sclerosis as well as in rheumatoid arthritis and psoriatic arthritis. This implies a possible benefit of antioxidant treatment. In scleroderma, however, only a few studies have been undertaken in this respect so far, and they yielded inconsistent outcomes (Denton et al 1999; Herrick et al 2000; Kalin et al 2002). The difficulty in assessing the benefit of antioxidant treatment lies probably in the subjectivity or insensitivity to change of most of the measures currently in use (Herrick & Matucci Cerinic 2001). Future trials focused on patients in earlier stages of systemic sclerosis, along with improved study design, could clarify the real clinical effect of antioxidant therapy. In the case of inflammatory arthritides, our data further support the opinion of clear benefit in adding antioxidants and micronutrients to conventional treatment, as has been confirmed by several studies in rheumatoid arthritis (Darlington & Stone 2001; Jaswal et al 2003), hence the same proposal can be offered also for psoriatic arthritis.

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List of abbreviations

AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABTS	2,2’-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)
AGE or AGEs	Advanced glycation end-products
ALEs	Advanced lipoxidation end-products
AMVN	2,2’-azobis(2,4-dimethylvaleronitrile)
AP-1	Activated protein 1
AST	Aspartate aminotransferase
cAST	Cytosolic aspartate aminotransferase
CAT	Catalase
CEL	N-ε-(carboxyethyl)lysine
CML	N-ε-(carboxymethyl)lysine
3-DG	3-deoxyglucosone
DOLD	Deoxyglucosone-lysine dimer
DPPH•	2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl radical
E_7	Reduction potential
EDRF	Endothelium-derived relaxing factor
ELISA	Enzyme-linked immunosorbent assay
FFI	2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole
Frc	Fructose
GC/MS	Gas chromatography/Mass spectrometry
GO	Glyoxal
GOLD	Glyoxal-lysine dimer
GPx	Glutathione peroxidases
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HCAs	Hydroxycinnamic acids
HClO	Hypochlorous acid
HPLC	High performance liquid chromatogaphy
HO•	Hydroxyl radical
4-HNE	4-hydroxy-2-nonenal
IDDM	Insuline dependent diabetes mellitus

IL-1	Interleukin-1
iNOS	Inducible nitric oxide synthase
LDL	Low density lipoprotein
MDA	Malondialdehyde
MGO	Methylglyoxal
MOLD	Methylglyoxal-lysine dimer
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro-blue tetrazolium
NF-κB	Nuclear factor kappa B
NIDDM	Non-insuline dependent diabetes mellitus
NO•	Nitric oxide
$^1O_2$	Singlet oxygen
O_2^{-}•	Superoxide anion radical
$ONOO^-$	Peroxynitrite
ORAC	Oxygen radical absorbance capacity
$ORAC_{ROO•}$	Peroxyl radical scavenging ability assessed by ORAC
PLP	Pyridoxal-5'-phosphate
PMN	Pyridoxamine-5'-phosphate
pI	Isoelectric point
PTB	Phenyl thiazolium bromide
PUFA	Polyunsaturated fatty acid
RAGE	Receptor for AGEs
RIA	Radioimmunoanalysis
RONS	Reactive oxygen and nitrogen species
ROO•	Peroxyl radical
ROS	Reactive oxygen species
SAR	Structure-activity relationships
SH	Sulfhydryl (thiol) group
SOD	Superoxide dismutase
sRAGE	Soluble receptor for AGEs
TEAC	Trolox equivalent antioxidant capacity
TNF-α	Tumor necrosis factor-α
VCAM-1	Vascular cellular adhesion molecule-1

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