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Supplementation with Gliadin-combined Plant Superoxide Dismutase Extract Promotes Antioxidant Defences and Protects Against Oxidative Stress

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The potential benefits to health of antioxidant enzymes supplied either through dietary intake or supplementation is still a matter of controversy. The development of dietary delivery systems using wheat gliadin biopolymers as a natural carrier represents a new alternative. Combination of antioxidant enzymes with this natural carrier not only delayed their degradation (i.e. the superoxide dismutase, SOD) during the gastrointestinal digestive process, but also promoted, *in vivo*, the cellular defences by strengthening the antioxidant status. The effects of supplementation for 28 days with a standardized melon SOD extract either combined (Glisodin®) or not with gliadin, were evaluated on various oxidative-stress biomarkers. As already described there was no change either in superoxide dismutase, catalase or glutathione peroxidase activities in blood circulation or in the liver following non-protected SOD supplementation. However, animals supplemented with Glisodin® showed a significant elevation in circulated antioxidant enzymes activities, correlated with an increased resistance of red blood cells to oxidative stress-induced hemolysis. In the presence of Sin-1, a chemical donor of peroxynitrites, mitochondria from hepatocytes regularly underwent membrane depolarization as the primary biological event of the apoptosis cascade. Hepatocytes isolated from animals supplemented with Glisodin® presented a delayed depolarization response and an enhanced resistance to oxidative stress-induced apoptosis. It is concluded that supplementation with gliadin-combined standardized melon SOD extract (Glisodin®) promoted the cellular antioxidant status and protected against oxidative stress-induced cell death. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords: antioxidant; plant superoxide dismutase; gliadin; oxidative stress.

INTRODUCTION

In all aerobic organisms, the consumption of oxygen is crucial for life. It also produces reactive oxygen species involved in the regulation of many different biological processes (Forman and Torres, 2002) and survival from invading pathogens. Under physiological conditions the production of these pro-oxidant molecules is controlled at different levels by the antioxidant defences that normally limit the excess of free radical species (Wei and Lee, 2002). These natural defences are essentially composed of specialized enzymes such as superoxide dismutase (SOD), catalase (Cat) and glutathione-peroxidase (Gpx) and also by non-enzymatic antioxidant molecules such as vitamins, thiols and β-carotene. Inflammatory or aging processes (Wickens, 2001) are associated with the disruption of the oxidant/antioxidant (redox) balance resulting in cellular and tissue oxidative stress and cell death by apoptosis (Lang *et al.*, 2002; Chandra *et al.*, 2000). Indeed, the progressive and discrete imbalance of the endogenous redox system can lead to the development of chronic degenerative diseases (Lavrovsky *et al.*, 2000; Tak *et al.*, 2000). Thus it seemed evident that nutritional antioxidant supplementation could have health-promoting effects if it could control the endogenous redox system (Fang *et al.*, 2002; Kritharides and Stocker, 2002). It is already admitted that dietary antioxidants are very useful in general health either by preventing or by supplementing the usual drug treatments in a variety of diseases (Stephens *et al.*, 1996; Kritchevsky, 1999; Burk, 2002). This suggests that the use of a nutritional antioxidant formula will provide better prevention of oxidative stress-mediated diseases.

Until now the development of these new functional foods has been limited by their poor capacity to promote efficient oral delivery of antioxidant enzymes and also by the definition of the correct health biomarkers to follow (Branca *et al.*, 2001). However, the

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development of new drug delivery and food packaging systems (Weber *et al.*, 2002; Takata *et al.*, 2002) make this new functional antioxidant formula possible (Mosca *et al.*, 2002; Stella *et al.*, 1995; Regnault *et al.*, 1996). Among various different delivery systems the wheat gliadin biopolymers presented a dual interest: (i) their capacity to trap and to delay the release of the active ingredient during the gastrointestinal digestive process (Arangoa *et al.*, 2001), and (ii) their bioadhesive properties with the intestinal mucosa to improve and/or promote the delivery of the active ingredient, thus defining an orally bioactive SOD (Dugas, 2002).

This study investigated the properties of an effective nutritional formula (Glisodin®) made from the combination of a melon (*Cucumis melo* LC.) standardized superoxide dismutase extract as the active ingredient and wheat (*Triticum vulgare*) gliadin biopolymers as the carrier. The antioxidant properties of the melon SOD contained in Glisodin® were evaluated on antioxidant biomarkers currently used to assess the potential health benefits of nutritional products.

MATERIALS AND METHODS

Reagents. Dulbecco's modified Eagle's medium (DMEM), L-glutamine, glucose, streptomycin-penicillin, fetal calf serum (FCS) and most of the chemical reagents were from Sigma Chemical Co (St Louis, MO). Hepatocytes were cultured in DMEM medium containing 10% FCS, 1% L-glutamine, 2% streptomycin-penicillin, in 5% CO_2 at 37 °C. The chemical donor nitrogen peroxide (Sin-1) was the kind gift of Dr J.P. Kolb (INSERM U311, Paris, France). 2,2′-azobis-(2-aminopropane)-dihydrochloride (AAPH) was obtained from Calbiochem (Meudon, France). Wheat gliadin (Gliamine®) was purchased from HITEX (Vannes, France). The standardized melon superoxide dismutase extract (Extramel®) was obtained from the strain *Cucumis melo* L.C., genetically selected for its higher grade SOD activity (90 IU/mg of dry powder), BIONOV (Avignon, France).

The gliadin-combined SOD preparation. Briefly, Glisodin® is a water dispersible form of superoxide dismutase lyophilized extract from melon (standardized to 90 IU/mg) combined with a 40% hydro-alcoholic soft gel of gliadin at 50 °C. It is spray-dried using maltodextrin as a support and the various ratios were adjusted to obtain a theoretical activity of 1 IU/mg of final dry powder.

The superoxide dismutase activity of the Glisodin® was certified using a specific enzymatic assay (Beauchamp and Fridovich, 1971; Oberley and Spitz, 1984) from 5 g of dry product sonicated into 7 mL of water. The solution was then centrifuged at 10 000 **g** for 20 min and the first supernatant (S1) made up to 10 mL with ultra pure water. The pellet was suspended again in 1 mL of ultra pure water, homogenized and centrifuged at 10 000 × **g** for 20 min at 6°–8 °C. The second supernatant (S2) was then adjusted to 1 mL. The activity in both fractions (S1 and S2) was determined on a native polyacrylamide gel electrophoresis against the SOD melon extract (90 IU/mg).

Delayed release of loaded SOD from the gliadin combination. The progressive release of the SOD activity trapped by the gliadin polymers was compared with the parallel degradation of the non-protected SOD (melon extract) during a process that mimicked the digestive transit (0.1 M hydrochloric acid at pH 1 in the presence of 1 μM of pepsin at 37 °C) as already described by Stella *et al.* (1995).

Animal population and treatment. Balb/c mice were purchased from IFFA-CREDO (Orleans, France), aged 6–8 weeks and weighing 25–30 g. Each group consisting of 10 animals randomly selected, received either a normal diet, or a supplementation with gliadin, or a supplementation with non-protected SOD melon extract (10 IU/day for 28 days) or Glisodin® (0.1, 0.5, 1, 5 mg/day for 28 days) by force-feeding.

Redox status. Blood samples were collected on heparin at different time-points along the supplementation period (0, 7, 14, 21 and 28 days). Plasma and erythrocytes were immediately separated by centrifugation at 800 × **g** for 20 min at 4 °C. Superoxide dismutase (RANSOD kit, Randox) glutathione peroxidase (RANSEL kit, Randox) and catalase activities (was assayed by a method in which the disappearance of peroxide is followed spectrophotometrically at 240 nm) were then determined. Red blood cell (RBC) hemolysis, induced by the free radical generator 2,2′-azobis-(2-amidinopropane)-dihydrochloride (AAPH), was determined as previously described (Miki *et al.*, 1987).

Peroxynitrite-induced apoptosis in hepatocytes. Apoptosis was quantified by using the ApoAlert DNA fragmentation detection kit (Clontech, Palo Alto, CA). At days 0, 7 and 28, hepatic cells were isolated and incubated for 48 h (5×10^5 cells/mL) in complete DMEM medium in the presence or in the absence of 100 ng/mL of Sin-1 (3-morpholinosydnonimine hydrochloride), a potent generator of nitrogen peroxide. Data are presented as the percentage of apoptotic cells among various areas of 200 cells.

Measurement of the mitochondrial depolarization, $\Delta\Psi_m$. The $\Delta\Psi_m$ of isolated hepatocytes was measured by flow cytometry using the J-aggregate-forming lipophilic cation, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) (Beltran *et al.*, 2000). Briefly, aliquots of the cell suspension (10^6 cells) were incubated with JC-1 at a final concentration of 3 μM at 37 °C in the dark for 30 min before analysis. Preliminary experiments demonstrated that under these conditions the dye reached near equilibrium distribution and gave a maximal fluorescence response to a fall in ΔΨm induced by the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (5 μM). Flow cytometry was performed on a FACScan instrument (Becton Dickinson). Data were acquired and analysed by using CELLQUEST software. The results are expressed as the mean aggregate fluorescence (red) alone.

Statistical analysis. Mean comparisons between the various groups (with or without supplemented diets) were conducted using Student's *t*-test. Differences were considered significant when $p < 0.05$).

Table 1. Effect of a supplementation with non-protected SOD on circulating antioxidants

Supplementation	SOD (U/g Hb)	Gpx (U/g Hb)	Catalase (kU/g Hb)
Control	1125 ± 55	798 ± 32	30 ± 2
Non protected SOD extract	1220 ± 40	810 ± 21	33 ± 6

Animals (n = 10) were fed every day with control diet supplemented or not with 10 mg/mouse/day of the non-protected SOD for 28 days. Blood samples were collected and SOD, Gpx and catalase activities were evaluated in erythrocytes. Data represent the mean ± SD of ten animals/group from one representative experiment.

RESULTS

Wheat gliadin carrier delays the SOD release in conditions mimicking the digestive process

Many investigations (Zidenberg-Cherr *et al.*, 1983; Giri and Misra, 1984) now including ours, demonstrated that oral treatment with non-protected SOD did not induce significant changes in the circulating redox status since the levels of erythrocyte SOD, catalase and Gpx activities remained constant (Table 1). This is consistent with the poor bioavailability or rapid degradation of proteins during the digestive process. As a matter of fact, a rapid disappearance of the non-protected SOD activity was observed in a medium mimicking the digestive process (Fig. 1) demonstrating that the antioxidant enzyme was destroyed during gastrointestinal transit. However, when the SOD activity was trapped by gliadin biopolymers (Glisodin®) a significant and progressive increase of SOD activity was observed probably correlating with the concomitant proteolysis of the gliadin biopolymers. This suggested that gliadin might delay the release and consequently the degradation of the SOD activity during gastrointestinal transit.

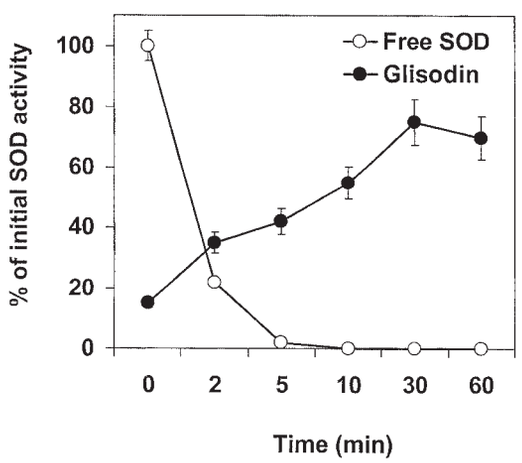

Figure 1. Gliadin polymers delay the release of the melon SOD activity in a medium mimicking the digestive process. An identical amount (100 units) of melon-SOD extract was submitted free or combined with gliadin (Glisodin®) to conditions mimicking the digestive process, for 1 h at 37 °C. The medium was periodically sampled to measure the residual SOD activity according to the reduction of ferricytochrome C. The data represent the mean ± SD of quadruplicate samples of one treatment out of six different experiments.

Table 2. Effect of a supplementation with SOD-gliadin combination on circulating antioxidants

	Supplementation	
	Control	Glisodin®
Antioxidant status (mmol/L)	1.39 ± 0.03	1.98 ± 0.06
SOD (U/g Hb)	1720 ± 125	3250 ± 255
Gpx (U/g Hb)	800 ± 33	1210 ± 89
Catalase (kU/g Hb)	35 ± 5	95 ± 6

Animals were fed every day with control diet or with control diet supplemented with 1 mg/mouse/day of Glisodin® for 28 days. Blood samples were collected and SOD, Gpx and catalase activities were evaluated in erythrocytes. Data represent the mean ± SD of ten animals/group from one representative experiment.

Glisodin® supplementation modulated the circulating antioxidant status

Supplementation of normal mice with the gliadin-combined standardized melon SOD extract (Glisodin®) for 28 days was found to promote the circulating antioxidant enzymes SOD, catalase and Gpx (Table 2). This effect was formula specific (Glisodin®), because the non-protected SOD extract or the gliadin alone was unable to promote these antioxidants. This promoting effect was time dependent (Fig. 2A) since the circulating SOD activity began to increase after 7 days of supplementation to reach a maximum after 28 days (SOD returned to the baseline after an additional 28 days, data not shown). The promoting effect was dose dependent (Fig. 2B) since significant effects appeared only for doses equivalent to 0.5 mg/day or higher with a maximal effect obtained at 5 mg/day.

As already demonstrated for different antioxidant dietary supplementation (Peng *et al.*, 2000), the supplementation with Glisodin® for 28 days increased the resistance of RBC ($p < 0.01$) to oxidative stress-induced hemolysis (Fig. 3) in response to a chemical donor of free radicals (AAPH). After 3 h of incubation at 37 °C in the presence of 50 mM AAPH, about 48% vs 74% of hemolysis was observed for RBC isolated, respectively, from animals supplemented or not with Glisodin®.

Hepatoprotective effect of Glisodin® supplementation

As previously described *in vitro* (Vouldoukis *et al.*, 2000), the SOD-gliadin combination also induced *in vivo*, a time-dependent increase in SOD activity in

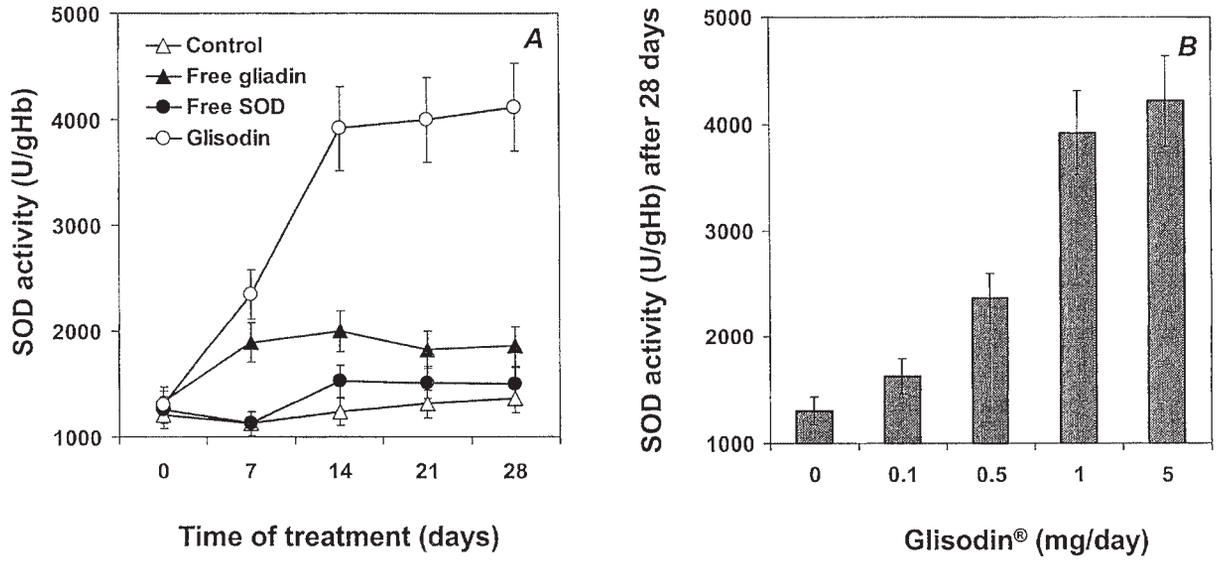

Figure 2. Effect of a supplementation with Glisodin® on circulating SOD activity. A. Mice were fed for 28 days, with either a control diet or supplemented with (a) melon SOD extract (10 IU of non protected SOD), (b) gliadin (1 mg) or (c) Glisodin® (1 mg for 1IU). B. Mice were fed with different doses of Glisodin® (0.1, 0.5, 1, or 5 mg of Glisodin®/mouse/day). Blood was periodically sampled in the study (A) while only at day 28 for study (B). SOD activity was measured as described in materials and methods. Data represent the mean ± SEM of the different groups.

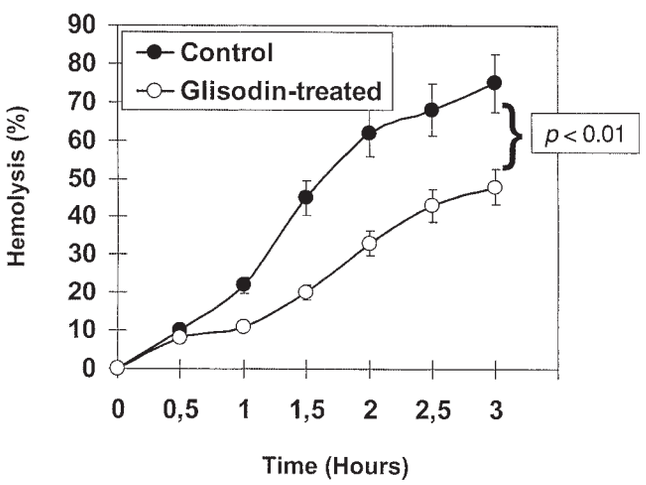

Figure 3. Effect of a supplementation with Glisodin® on erythrocyte resistance to oxidative stress-induced hemolysis. After a 28 day period of supplementation with Glisodin® (1 mg/day), RBC were collected and exposed to the free radical generator AAPH (50 mM). Hemolysis was evaluated as described in materials and methods. Data represent the mean ± SEM of the different groups.

hepatocytes (Fig. 4). This inducing effect appeared to be significant ($p < 0.05$) after 14 days of Glisodin® supplementation and reached a maximal effect after 21–28 days ($p < 0.001$). Such stimulation was not restricted to the SOD activity because catalase and Gpx activities were also increased (Table 3). As shown in Fig. 5 the improvement of the hepatocyte antioxidant defences correlated with an increased resistance ($p < 0.01$) to oxidative stress-induced apoptosis (Estevez and Jordan, 2002). After 8 h in the presence of the peroxynitrites chemical donor Sin-1, it was observed that 20% of the hepatocytes isolated from animals supplemented with Glisodin® underwent apoptotsis, whereas this rate increased to 72% in hepatocytes from untreated animals.

Table 3. Effect of a supplementation with SOD-gliadin combination on liver antioxidants

	Activity (unit/mg of protein)		
Supplementation	SOD	Gpx	Catalase
Control	2.5 ± 0.2	0.21 ± 0.05	40 ± 1
Glisodin®	13.5 ± 0.6	0.80 ± 0.02	68 ± 3

Animals received every day either a control diet with or without supplementation with 1 mg/mouse/day of Glisodin® for 28 days. Livers were then collected and then the SOD, catalase and Gpx activities were evaluated from the various tissue extracts. Data represent the mean ± SD of ten animals/group.

Effect of Glisodin® supplementation on animal hepatocytes mitchondrial $\Delta\Psi_m$ exposed *ex vivo* to Sin-1

As the mitochondrion is a key compartment involved in the control of oxidative stress-induced cell death (Akao *et al.*, 2003a) the mitochondrial functions of hepatocytes isolated from animals receiving a Glisodin® supplementation were evaluated. As already described (Li *et al.*, 2002; Kahlert and Reiser, 2002; Makani *et al.*, 2002) mitochondria from normal hepatocytes exposed to Sin-1 showed a gradual decrease in $\Delta\Psi_m$ as described by the mean aggregate fluorescence of the cationic lipophilic fluorochrome (JC-1) (Fig. 6). Analysis of mitochondrial $\Delta\Psi_m$ of Sin-1-stimulated hepatocytes from Glisodin® supplemented animals demonstrated that the mitochondrial depolarization was substantially delayed.

DISCUSSION

This study investigated the potential effects of a supplement containing a gliadin-combined plant SOD

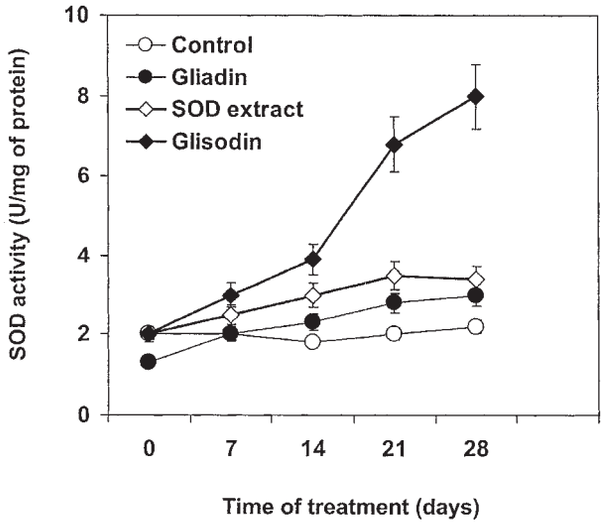

Figure 4. Effect of a supplementation with Glisodin® on liver SOD activity. Mice were fed with a control diet supplemented or not with 1 mg/mouse/day of Glisodin®. Animals were killed periodically each 7 days. Liver proteins were extracted and the SOD activity was evaluated. The results are expressed as units per mg of protein and data represent the mean ± SEM of the different groups.

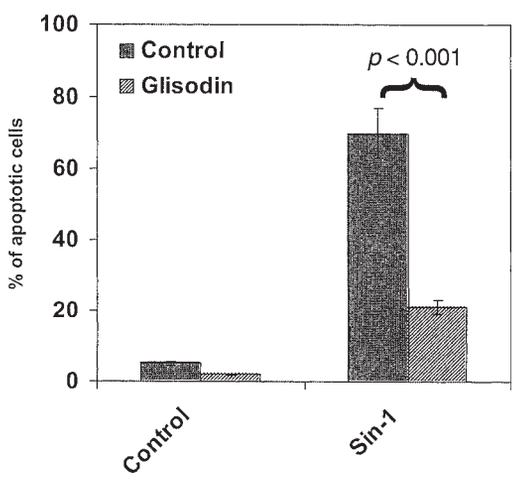

Figure 5. Effect of a supplementation with Glisodin® on the resistance of hepatocytes to nitrogen peroxide-induced apoptosis. Balb/c mice (n = 10 per group) were fed a control diet supplemented or not with 1 mg/mouse/day of Glisodin® and killed after 28 days. After isolation liver cells were submitted to Sin-1. The results are expressed as a percent of apoptotic cells and data represent the mean ± SEM of four different experiments.

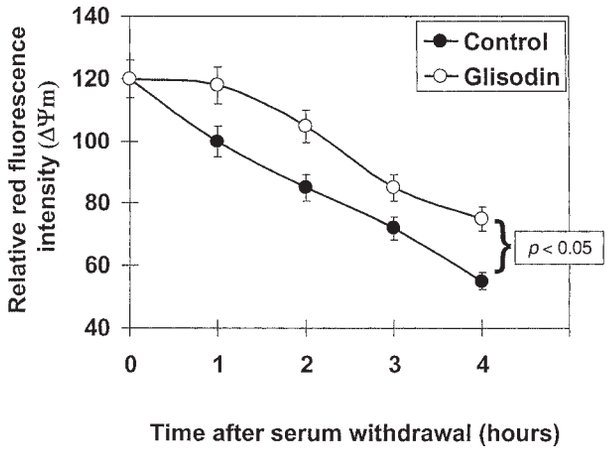

Figure 6. Effect of a supplementation with Glisodin® on Sin-1-induced mitochondrial membrane depolarization $\Delta\Psi_m$ in hepatocytes. Changes in $\Delta\Psi_m$ of isolated hepatocytes from normal or Glisodin® (n = 10 per group) supplemented animals were followed after exposure to the chemical peroxynitrite donor, Sin-1 (100 ng/mL) over a period of 4 h as described in materials and methods. Data represent the mean ± SD of all animals.

extract on several redox biomarkers. The results of this animal study were dual: the Glisodin® dietary supplementation not only promoted the circulating and tissue antioxidant defences (increased SOD, Gpx and catalase activities) but also improved cell resistance to oxidative stress. In the circulation, RBC from animals receiving Glisodin® were less susceptible to oxidative-stress-induced hemolysis. In addition hepatocytes from animals receiving Glisodin® dietary supplementation were resistant to peroxynitrite-induced apoptosis and mitochondrial depolarization.

The combination of the melon SOD extract with gliadin biopolymers is mandatory for obtaining this health promoting effect, confirming that the wheat gliadin is a helpful carrier for the oral delivery of active food ingredients (Arangoa *et al.*, 2001).

Many studies have reported that a long-lasting intake of fruit and vegetable antioxidants reduced the likelihood of cardiovascular and proinflammatory diseases as well as certain cancers (Block *et al.*, 1992; Diplock *et al.*, 1987; Madar and Stark, 2002; O'Byrne *et al.*, 2002; Akao *et al.*, 2003b). So it appears that the improvement of antioxidant defences is a biological key event in the health promoting effects of antioxidant nutrients. The present work not only confirms and extends these scientific and clinical studies but also provides useful information for the development of functionally active food ingredients.

This new formula shows real benefits for health since functional antioxidant enzyme supplementation (here melon SOD) is now able to promote cellular resistance to stress by strengthening the host antioxidant defences. Nevertheless, the mechanism by which it exerts its biological effect remains to be clarified.

The present study does not only confirms the efficacy of dietary antioxidant supplementation but also describes an orally active plant superoxide dismutase demonstrating that functional enzymes can be used in dietary supplementation.

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