African Journal of Biotechnology Vol. 11(4), pp. 938-949, 12 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1741
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Improvement of antioxidant potential in rats consuming feathers protein hydrolysate obtained by fermentation of the keratinolytic bacterium, *Bacillus pumilus* A1

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Accepted 4 November, 2011

The *in vitro* and *in vivo* antioxidant activities of feathers protein hydrolysate (FPH), produced by fermentation with the keratinolytic bacterium, *Bacillus pumilus* strain A1, were evaluated. The antioxidant activities of FPH, evaluated using DNA nicking and ferrozine assays, demonstrate that FPH present an important antioxidant potential. Interestingly, the addition of this hydrolysate (2.5 and 5%) to the feed of rats caused a significant decrease in the rate of thiobarbutiric acid reagent substances (TBARS) and the enzymatic antioxidants activities (superoxide dismutase, glutathione peroxidase and catalase) in liver, kidney, heart, brain and cerebella, as compared to the control. However, the supplementation of the standard food by 2.5% of untreated feather meal or 5% of soya is associated with an increase of the TBARS rate and enzymatic antioxidant activities. These results indicate that FPH may be useful as supplementary proteins and natural antioxidant in animal feed formulation.

Key words: Feather protein hydrolysate, *Bacillus pumilus*, lipids peroxidation, oxidative stress, dietary ingredient.

INTRODUCTION

Feathers are produced in large amounts as a waste by-product at poultry-processing industries, reaching millions of tons annually throughout the world. Feathers, consisted mainly of keratin characterized by its high recalcitrant nature, could be an important protein source in animal feedstuff. In fact, keratinous wastes were efficiently bioconverted by keratinolytic microorganisms (Dastager et al., 2009, Ouled Haddar et al., 2009, Fakhfakh et al., 2009, Kumar et al., 2010). The resulting hydrolysate was characterized by a high nutritional value as compared to feathers converted by classic physicochemical treatments (Papadopoulos et al., 1986). Consequently, the feathers hydrolysate can serve as protein complements in animal feed (Coward et al., 2006, Grazziotin et al., 2008) and fish aquaculture (Bishop et al., 1995).

Fish feeds containing high levels of polyunsaturated fatty acids, were protected against lipid oxidation in the presence of synthetic antioxidants, mainly ethoxyquin and butylated hydroxytoluene (BHT) (Hamre et al., 2010). Recently, there has been a substantial carry-over of these antioxidants to the fish fillet and the mandatory two weeks starvation period before slaughter of farmed fish is not sufficient for clearance of these antioxidants from the fillet (Bohne et al., 2008, Hamre et al., 2010). Therefore, a switch to natural antioxidants in fish feed ingredients would be an advantage both for the aquaculture industry, and with regard to consumer health and well-being. Furthermore, the relationship between nutrition and animal health has long been recognized. Animal's morbidity and mortality can be explained partly by impaired immune responsiveness. Consequently, the role of dietary natural antioxidants in immunity and health of animals is of prime importance (Chew, 1996).

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Table 1. Physico-chemical composition of the standard food (%).

Parameter	Value
Moisture	14
Fibres	5
Protein	20
Fat	3
Ash	13.5

A number of oxygenated compounds were produced during the attack of free radicals against membrane lipoproteins, proteins and polyunsaturated fatty acids. One of them is malondialdehyde (MDA) which could be used as an indicator of oxidative stress, as its concentration in plasma increased as a result of free-radical processes. The antioxidative system enables transformation of reactive oxygen species (ROS) into inactive and harmless compounds. Natural antioxidant enzymes such as superoxide-dismutase (SOD), glutathione peroxidase activity (GPx) and catalase (CAT) provided primary defense against ROS. SOD could selectively scavenge a superoxide radical by catalysing its dismutation to hydrogen peroxide and molecular oxygen, while GPx and CAT served to decompose hydrogen peroxide to the unreactive species. Therefore, *in vivo* studies could provide valuable information on how dietary antioxidants can protect against oxidative damage in some tissues (Farombi et al., 2004, Pasko et al., 2010).

To the best of our knowledge, this is the first report highlighting the *in vivo* antioxidant potential of a keratinous waste hydrolysate. The effect of dietary supplementation with feathers protein hydrolysate, feathers meal and soya on corporal mass and oxidative status of Wistar rats was investigated.

MATERIALS AND METHODS

Feather meal

Chicken feathers, supplied by a local poultry industry (Chahia, Tunisia), were washed 3-fold with tap water and then with distilled water. The washed feathers were then cooked until they were boiled for 20 min and pressed to remove water. The resulting pressed product was minced using a blender, dried at 90°C for 22 h, minced again and sifted to obtain a fine powder, and stored at room temperature.

Bacterial strain

B. pumilus A1 producing alkaline keratinases was isolated from a slaughter house polluted water in Sfax city (Tunisia). It was identified on the basis of the 16S rRNA gene sequencing (EU719191) (Fakhfakh-Zouari et al., 2010).

Preparation of feather protein hydrolysate

The strain *B. pumilus* A1 was routinely grown in Luria–Bertani (LB) broth medium composed of (g/L): peptone, 10; yeast extract, 5; and NaCl, 5 (Miller, 1972). The medium used for feather protein hydrolysate (FPH) production was composed of (g/L) chicken feather, 50; KH_2PO_4, 0.5; K_2HPO_4, 0.5; NaCl, 2.0; KCl, 0.1; and $MgSO_4$ (7 H_2O), 0.1; pH 10.0. The media were autoclaved at 121°C for 20 min. Cultivations were conducted in a 1 L Erlenmeyer flask containing 100 ml culture medium maintained for two days at 45°C and 250 rpm. After incubation, the culture was autoclaved and dried overnight at 90°C. The product was hammer milled to reach 1 mm mesh screen and stored at room temperature until used.

In vitro antioxidant activity test

DNA nicking assay

DNA nicking assay was performed using pCRII™TOPO plasmid (*invitrogen*) by the method of Lee et al. (2002) with slight modifications. A mixture of 10 µl of FPH at a concentration of 2 mg/ml and plasmid DNA (0.5 µg/well) were incubated for 10 min at room temperature followed by the addition of 10 µl of Fenton's reagent (30 mM H_2O_2, 50 µM L-ascorbic acid and 80 µM $FeCl_3$). The mixture was then incubated for 5 min at 37°C. The DNA was analysed on an agarose gel (1%).

Determination of metal (Fe^{2+}) chelating activity of FPH (ferrozine assay)

The chelating activity of the FPH for Fe^{2+} was measured according to the methods described by Dinis et al. (1994). To 0.5 ml of FPH, 1.6 ml of deionised water and 0.05 ml of $FeCl_2$ (2 mM) was added, followed by the addition of 0.1 ml of ferrozine (5 mM) after 15 min. After 10 min at room temperature, the absorbance of the Fe^{2+} - ferrozine complexes with red or violet colour was measured at 562 nm. The chelating anti-oxidant activity for Fe^{2+} was calculated according to the following formula:

Chelating rate (%) = (Ac – As) / Ac × 100

Where, Ac is the absorbance of the control reaction and As is the absorbance of the sample extract.

Animals and treatment

Adult male Wistar rats (n = 30), weighting 190 ± 2 g, were purchased from Central Pharmacy, Tunisia. Animals were acclimatized for seven days at room temperature (22 ± 2°C) with a 10 h of darkness and 14 h of light in a room under hygienic conditions. They were fed with a standard diet supplied by the Society of Animals Nutrition, Sfax, Tunisia. The standard diet was composed of wheat, sound, lucerne, soya, oil and vitamins minerals compound (VMC). The physico-chemical composition of the standard food is illustrated in Table 1. The rats were randomly divided into five groups containing six rats each and caged separately. Animals of one group remained on the standard diet and were considered as control animals. The other groups of animals were switched to the treatment diet containing 2.5% FPH, 5% FPH, 2.5% feather meal (FM) and 5% soya, for 15 days before slaughtering. The animals were sacrificed on the morning of the 16th day and the organs (liver, kidney, heart, brain and cerebellum) were separated, washed and weighed. 1 g of each rat organ was cut into small pieces and immersed into 2 ml of ice-cold lysis buffer (TBS : Tris 50 mM, NaCl 150 mM, pH 7,4), then sonicated (for 10 s, twice) and centrifuged (5000 *g*, 30 min, 4°C). Organ extracts were collected and stored at -80°C for the biochemical analysis.

Biochemical analysis

Estimation of lipid peroxidation

Lipid peroxidation was estimated as evidenced by the formation of the TBARS. TBARS were assayed in tissues by the method described by Yagi (1976). MDA and other TBARS were measured by their reactivity with thiobarbituric acid (TBA) in an acidic condition to generate pink coloured chromophore which was read at 530 nm.

375 µl of organ extracts were homogenized with 150 µl of TBS and 375 µl of BHT in order to precipitate proteins and centrifuged (1000 g, 10 min, 4°C). 400 µl of supernatant were mixed with 80 µl of HCl (0.6 M) and 320 µl of Tris-TBA (Tris 26 mM ; TBA 120 mM), and the mixture was heated for 10 min at 80°C. The absorbance of the resultant supernatant was read at 530 nm. The amount of TBARS was calculated using an extinction coefficient of $1.56\ 10^5\ mM^{-1}cm^{-1}$.

Enzyme assays

SOD activity was assayed by measuring its ability to inhibit the photo reduction of nitroblue terazolium (NBT) (Asada et al., 1974). In this assay, one unit of SOD is defined as the amount required inhibiting the photo reduction of NBT by 50%. 50 µl of organ extract was combined with 1 ml of EDTA-methionine (0.1 mM EDTA ; 13 mM methionine), 842.2 µl of 50 mM phosphate buffer (pH 7.8) and 85.2 µl of NBT solution (2.64 mM prepared in phosphate buffer 50 mM). Riboflavin was added to a final concentration of 0.26 mM and switching on the light started the reaction. The absorbance was read at 580 nm after incubation for 20 min.

The activity of catalase was assayed by the method of Aebi, (1974). The reaction mixture contained 780 µl of 1 M phosphate buffer, 200 µl of 500 mM H_2O_2 and 20 µl of the supernatant. The reaction started by adding H_2O_2 and its decomposition was monitored directly by the decrease in absorbance at 240 nm for 1 min. The enzyme activity was calculated using an extinction coefficient of $0.043\ mM^{-1}cm^{-1}$.

GPx activity was assayed by the method of Flohe and Gunzler (1984). 200 µl of organ extract was combined with 400 µl of glutathione (GSH) (0.1 mM) and 200 µl of $KNaHPO_4$ (67 mM). The mixture was incubated for 5 min at 25°C. After that, 200 µl of H_2O_2 (1.3 mM) was added to initiate the reaction. After 10 min, the reaction was stopped by the addition of 1 ml 1% (w/v) trichloroacetic acid and was allowed to rest for 30 min at room temperature, and then it was centrifuged for 10 min at 3000 rpm. After a specific time, the remaining GSH content was measured by Ellman's method (1959). 480 µl of the supernatant was mixed with 2200 µl of Na_2HPO_4 (0.32 M) and 320 µl of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB, 1 mM). The yellow colour developed was read at 412 nm.

The protein rate was determined by Lowry et al. (1951) using the bovine serum albumin as standard.

Statistical analysis

Means ± SEM were calculated for the different groups of rats. The statistical evaluation of the data was achieved using Student's t-test as described by Garret (1956). Statistical significance was set at $p \leq 0.05$.

RESULTS

In vitro studies

At first, the *in vitro* antioxidant activity of FPH was assayed using DNA nicking assay and ferrozine assay.

DNA damage protection potential assay

Hydroxyl radical is the most reactive free radical. It has the highest 1-electron reduction potential (2310 mV) (Korycka-Dahl and Richardson, 1978) and can react with lipids, polypeptides, proteins and DNA, especially thiamine and guanosine (Ashok and Ali, 1999). In order to test DNA damage protection potential of FPH, the effect of FPH at a concentration of 2 mg/ml on DNA cleavage was investigated.

Hydroxyl radicals generated by the Fenton reaction are known to cause oxidative induced breaks in DNA strands to yield its open circular or relaxed forms. Figure 1a shows the agarose gel electrophoresis pattern of plasmid DNA incubated with Fenton's reagent in the absence and presence of FPH. Lane 1 shows an untreated plasmid with its two forms: the upper one is the open-circular (nicked) DNA and the faster migrating band corresponded to supercoiled (closed circular) plasmid. Incubation of plasmid DNA with Fenton's reagent in the absence of FPH resulted in the complete degradation of the two DNA bands (native supercoiled circular DNA and open circular form) (lane 2). This DNA damage was reduced in the presence of FPH. In fact, in the presence of 1 mg/ml, the FPH showed a moderate protection to supercoiled plasmid and a complete protection to the open-circular (nicked) DNA.

Metal (Fe^{2+}) chelating activity

The metal-chelating activity is also widely used in evaluating the antioxidant activity of different natural products. Transition metal ions can stimulate lipid peroxidation by two mechanisms, namely by participating in the generation of initiating species and by accelerating peroxidation decomposing lipid hydroperoxides into other components which are able to abstract hydrogen, perpetuating the chain of reaction of lipid peroxidation (Deshpande et al., 1995). The dark color complex formed by the interaction of ferrozin with Fe^{2+} ions was decreased by the action of metal chelator compounds that exist in the reaction mixtures. Data presented in Figure 1b shows the chelating activity of FPH at different concentrations (0.5 to 6 mg/ml) compared with ethylenediaminetertracetic acid (EDTA) as positive standard. The results show that the percentages of inhibition of the ferrozine–Fe^{2+} complex formation increased linearly with the FPH concentration. Although the chemical EDTA exhibited the highest antioxidant activity, natural antioxidants are of growing interest.

In vivo study

In this study, feed of rats was supplemented with FPH at

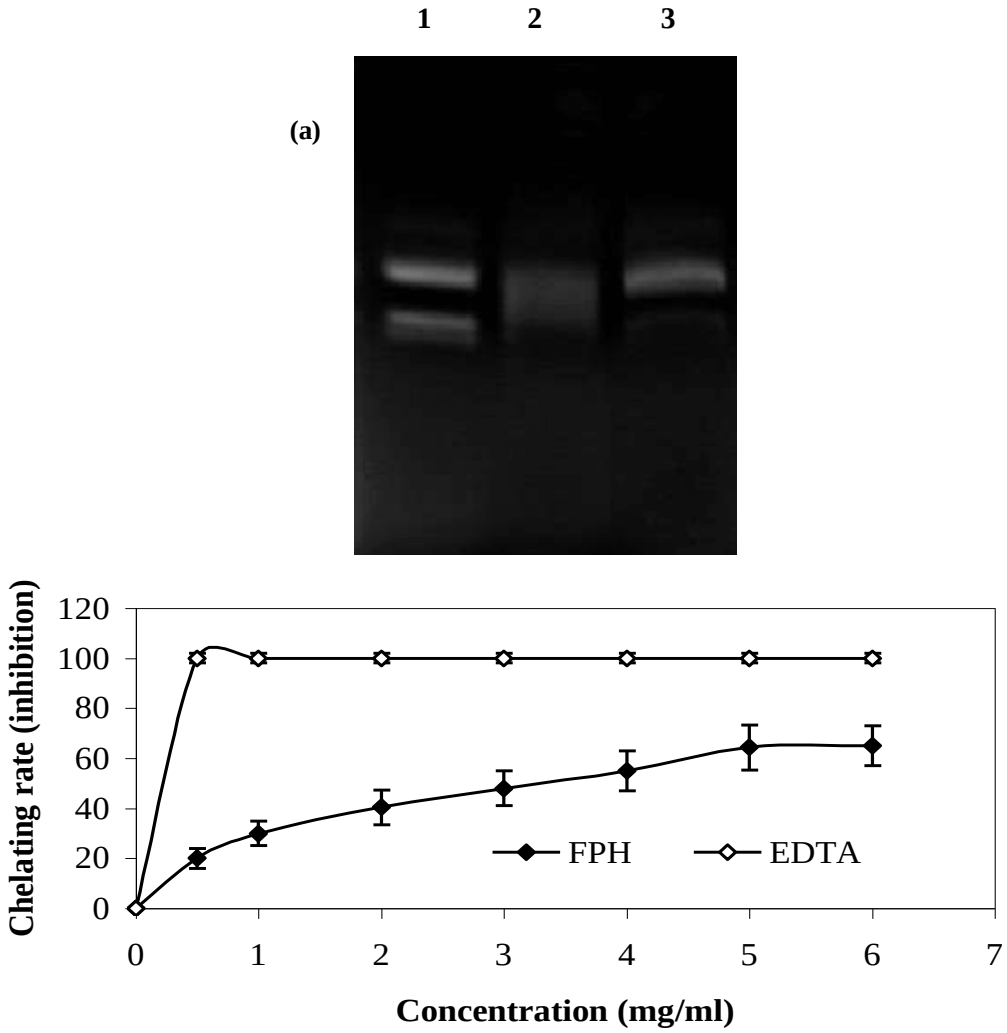

Figure 1. *In vitro* antioxidant activity of feathers protein hydrolysate (FPH). (a) DNA damage protection potential assay. Lane 1, untreated plasmid; lane 2, plasmid DNA with Fenton's reagent; lane 3, plasmid DNA with Fenton's reagent in the presence of FPH. (b) Metal (Fe^{2+}) chelating activity.

concentrations of 2.5 and 5% for 15 days before slaughtering. Feather meal (2.5) and soya (5%) which represented no hydrolyzed protein sources were also tested.

No mortality and no evident signs of neurotoxicity (trembling, respiratory difficulties) were recorded in all the treated rats.

Effect of the FPH on corporal and organs mass

Live body mass was recorded during animal raising. As represented in Figure 2, the incorporation of 2.5 or 5% FPH in the rats' diet resulted in a slight increase of the body mass compared with control rats fed with a standard food (Figure 2). Furthermore, the increase of rats' body mass was accompanied by an increase of the mass of the various organs compared to control rats (Figure 3). However, the addition of 5% soya or 2.5% FM to the rats' diet resulted in an important decrease of corporal and all organs masses with regard to the control diet (Figure 2, 3). Thus, the use of feather hydrolysate favors a better growth of rats.

Impact of the treatment on the lipid peroxydation

Lipids of living bodies are particularly sensitive to the oxidation, especially those formed by polyunsaturated fatty acids in cellular membranes (Halliwell et al., 1992). So, numerous products are generated during the lipids peroxydation most of which are mutagens. Among these products, considered as markers of this peroxydation, we can quote MDA which results from the fragmentation of the polyunsaturated peroxide fatty acids, the thiobarbutiric acid, hydrocarbons with short chains and hydroxy-

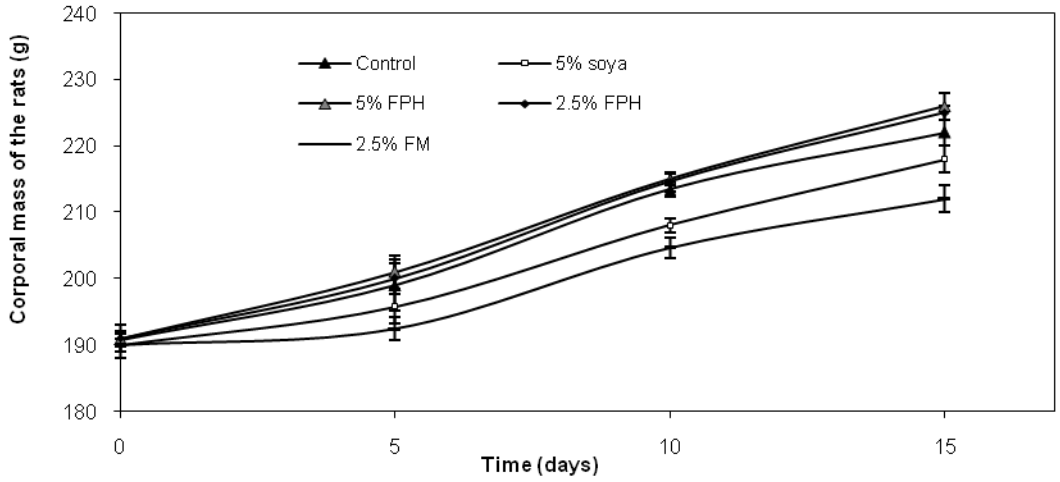

Figure 2. Evolution of the corporal masses of rats fed with various sources of protein.

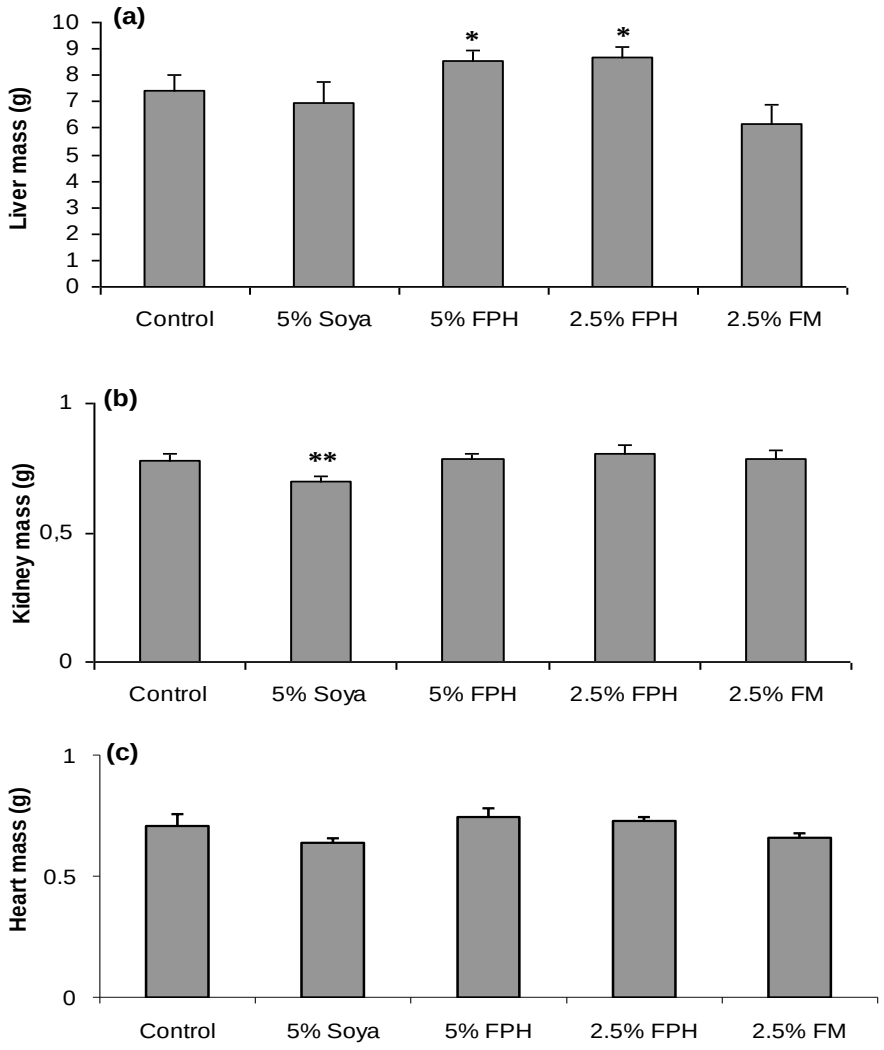

Figure 3. Effect of various sources of protein on the mass of liver (a) kidney (b) heart (c) brain (d) and cerebellum (e). **: p≤ 0.01; *: p ≤ 0.05 as compared with the control.

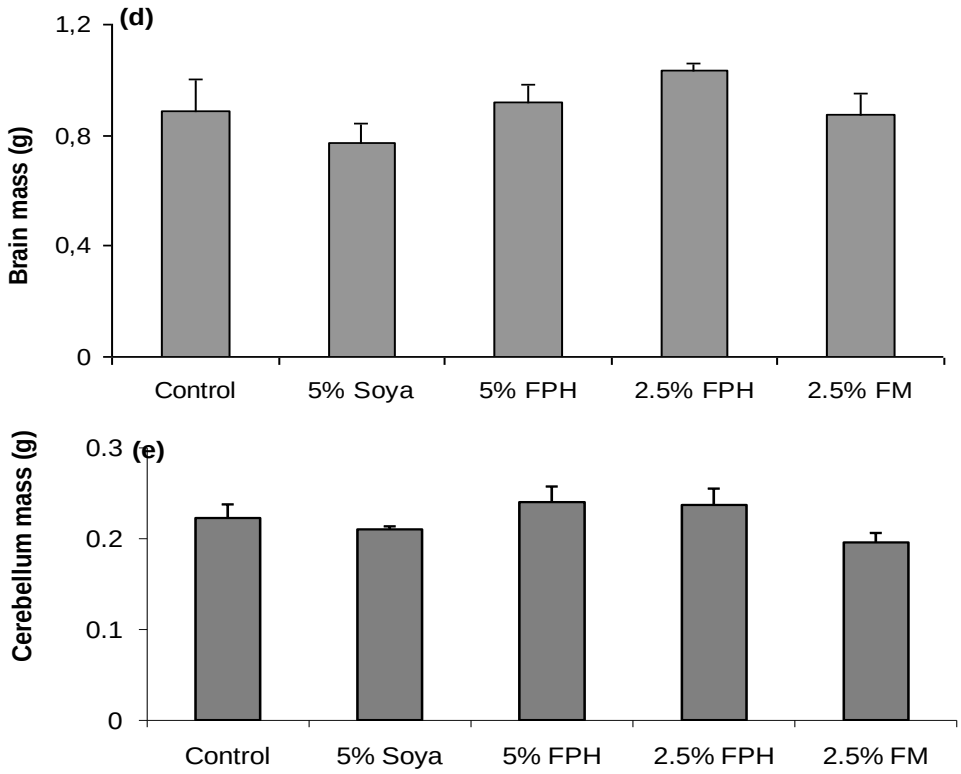

Figure 3. Contd.

alkenal (Sahnoun et al., 1997).

The consumption of 5% soya or 2.5% FM provoked a significant increase of TBARS level in all organs, which indicate the oxidative effect of these treatments (Figure 4). However, the administration of FPH in the food of rats significantly decreased the peroxydatif effect in their organs at levels lower than those of the control rats (Figure 4). This indicates the beneficial effect of FPH against the oxidative stress and confirms the *in vitro* antioxidant activity of the FPH.

Impact of the treatment on the antioxidant enzymatic system

In vivo, a variety of enzymatic antioxidants, such as catalase, superoxide dismutase and glutathione peroxidase provide a primary defence against reactive oxygen species (Dimitrios, 2006). Measurements of these enzymes activities in various organs extracts is an interesting tool to evaluate the effect of FPH, soya and FM on the oxidative status of rats.

Impact of the treatment on SOD

The administration of 5% soya or 2.5 % FM in the rats' food resulted in a significant increase of the SOD activity in all the organs compared with control rats (Figure 5). This testifies the increase of the free radicals and the establishment of an oxidative stress by the ingestion of unhydrolysed proteins.

However, the addition of 2.5 or 5% of FPH in the food significantly decreased the SOD activity, which showed the beneficial effect of this hydrolysate as antioxidant and confirms the antioxidant activity attributed to the FPH.

Impact of the treatment on the catalase activity

As shown in Figure 6, there was a significant increase in the catalase activity to rats treated with 5% soya or 2.5 % FM compared with control z rats. Nevertheless, the addition of FPH in the food of rats showed a significant decrease in the catalase activity. This decrease confirms the presence of antioxidant activity in the FPH associated with the presence of the small peptides.

Impact of the treatment on the GPx

The measure of the GPx activity showed a significant increase of the production of this enzyme in the liver, the kidney, the heart, the brain and the cerebellum of rats fed with 5% soya or by 2.5 % FM compared with control rats (Figure 7). The rats fed with feed supplemented with 2.5 or 5% FPH resulted in a significant decrease in the

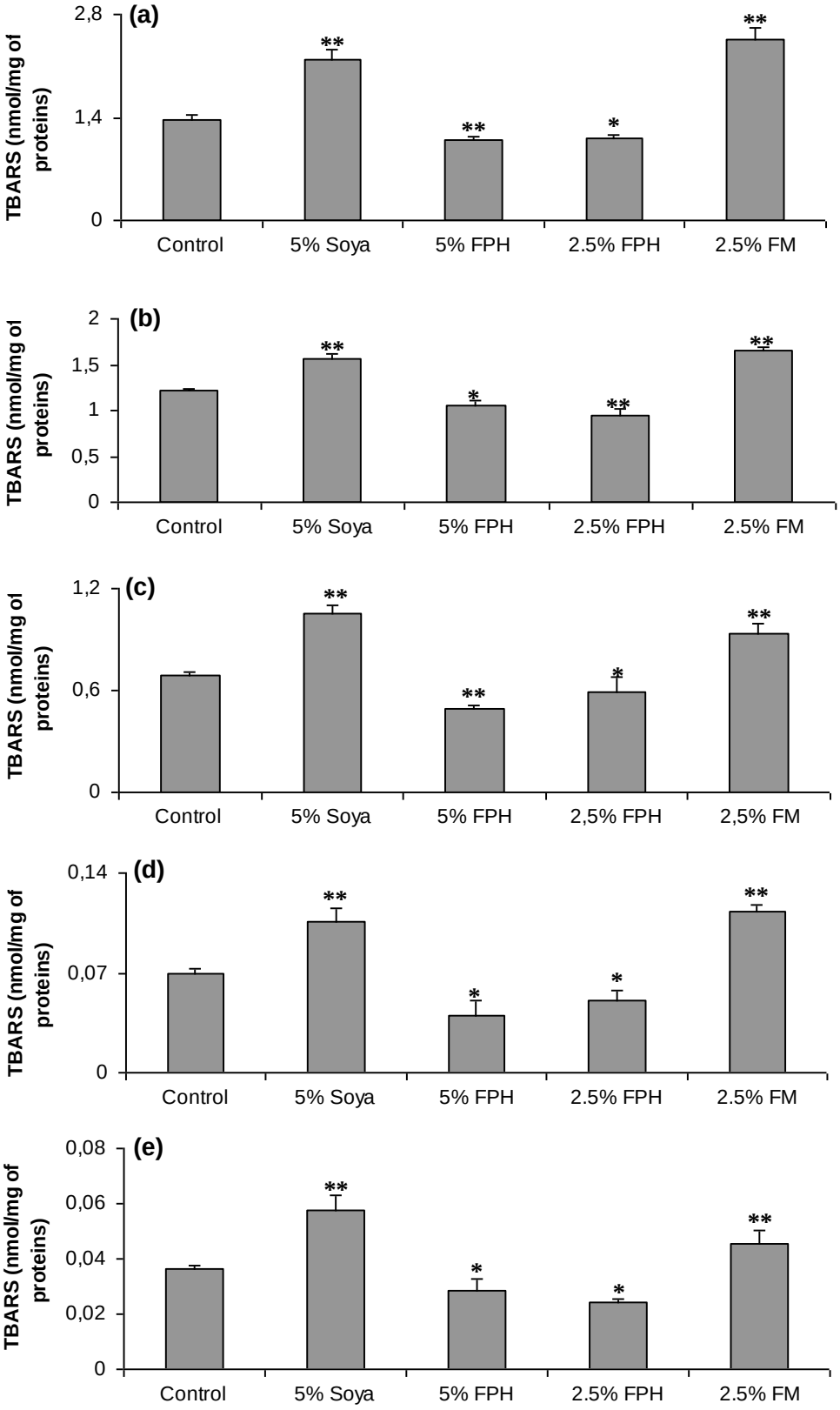

Figure 4. Impact of the treatment on the levels of TBARS (nmol/mg of proteins) on (a) liver (b) kidney (c) heart. (d) brain and (e) and cerebellum. **p ≤ 0.01; *p ≤ 0.05 as compared with the control.

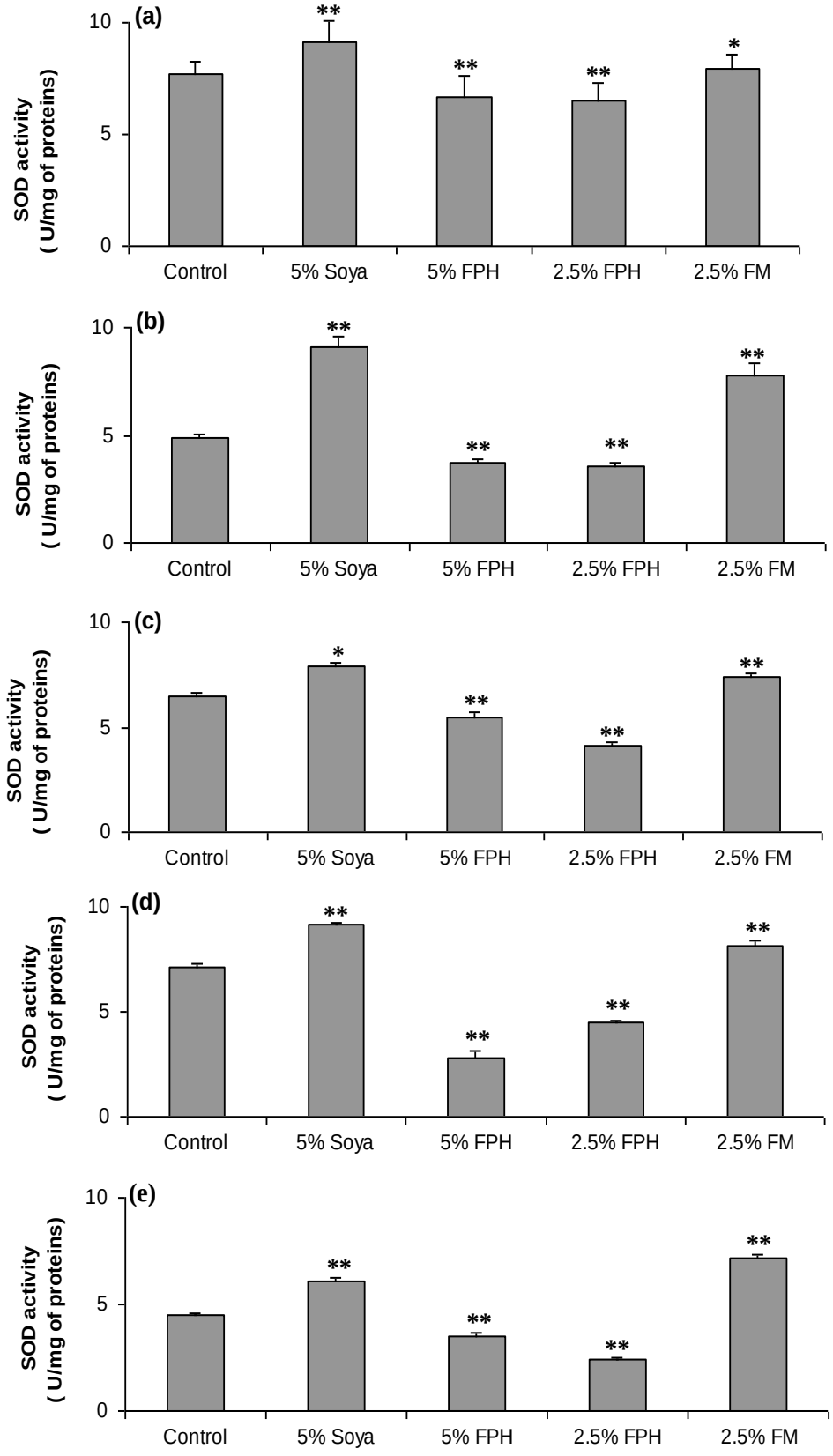

Figure 5. Impact of the treatment on SOD on (a) liver (b) kidney (c) heart (d) brain and (e) cerebellum. **p ≤ 0.01; *p ≤ 0.05 as compared with the control.

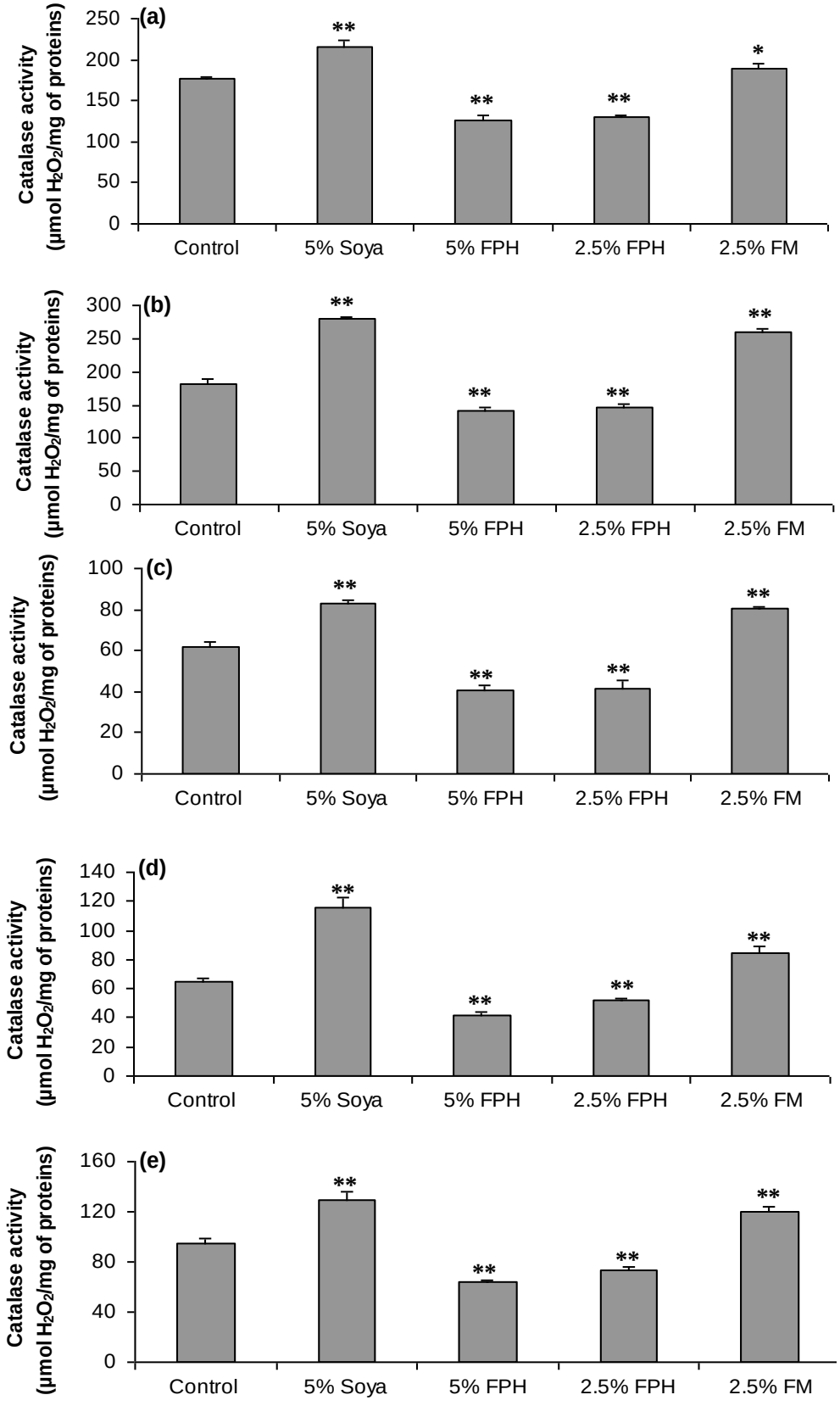

Figure 6. Impact of the treatment on the catalase activity on (a) liver (b) kidney (c) heart (d) brain and (e) and cerebellum. **p ≤ 0.01; *p ≤ 0.05 as compared with the control.

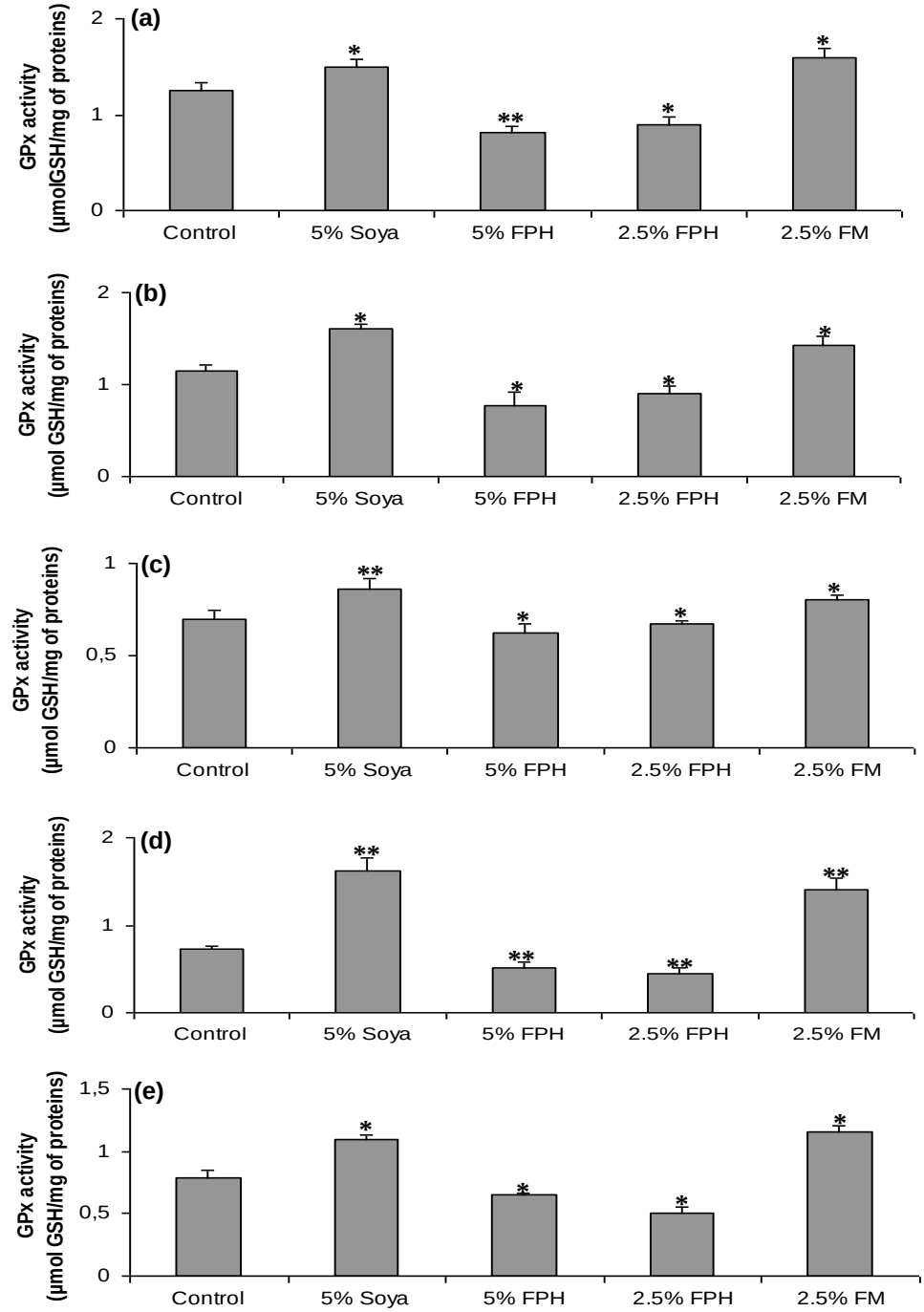

Figure 7. Impact of the treatment on the glutathion peroxydase activity (GPx) on (a) liver (b) kidney (c) heart (d) brain and (e) cerebellum. **p≤ 0.01; *p ≤ 0.05 as compared with the control.

production of the GPx activity.

DISCUSSION

Microbial conversion of keratinous wastes constitutes a potential technique for their biodegradation and valorisation with respect to cost-effectiveness and environment protection (Shih, 1993; Dalev, 1994). In this work, a feather-degrading bacterium, *B. pumilus* A1 was used to produce FPH, which was examined for its capacity to

contain metabolisable protein and antioxidant peptides.

The *in vitro* antioxidant activity of FPH was demonstrated using DNA nicking and ferrozine assays. In fact, the FPH effectively mitigates the oxidative stresses on susceptible biomolecules, such as DNA. A similar result was obtained with antioxidant peptides from bullfrog muscle protein hydrolysate (Je et al., 2007). In addition, the FPH exhibited a strong inhibition of ferrozine–Fe^{2+} complex formation. This was probably due to the presence of peptides in FPH which can act as excellent metal chelators. A similar result was reported by Chan and Decker (1994), who showed that the antioxidant activity of meat dipeptide carnosine was due to its metal chelating activities. Furthermore, the hydrolysates obtained from porcine myofibrillar proteins by treatment with papain or actinase E possessed 1, 1-diphenyl-2-picrylhydrazyl radical scavenging activity and chelating activity toward metal ions (Saiga et al., 2003).

Oxidation in foods constitutes one of the major causes of their deterioration (Antolovich et al., 2002). Utilisation of synthetic antioxidants, such as butylated hydroxyanisole, BHT and propyl gallate to retard lipid oxidation is regulated because of the possible health risks associated with their use (Bohne et al., 2008; Hamre et al., 2010). For this reason, the replacement of synthetic antioxidants with natural antioxidants could have beneficial effects in terms of health implications (Moure et al., 2001). Thus, the FPH can be used as a natural antioxidant for the protection of food against deterioration.

In terms of foods, antioxidants are compounds which are able to delay, retard or prevent auto-oxidation processes (Mielnik et al., 2003). However, in terms of the effects in the animal body, an antioxidant can be defined as ‘‘a substance that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen, on normal physiological functions” (Huang et al., 2005). Therefore, it is necessary to perform *in vivo* assays to ensure that the *in vitro* antioxidant activity of FPH are bio-available following ingestion and can reach a target site and administer a response in a living system. The FPH was incorporated in the diet of rats to study its effect on their physical growth and on the *in vivo* lipid peroxidation.

The rats fed with feed supplemented with 2.5 or 5% FPH resulted in a slight increase of the body mass compared with control rats and with rats fed with a food supplemented with 5% soya or 2.5% FM. In agreement with these results, feather hydrolysate obtained with *Vibrio* sp. kr2 strain showed better *in vitro* nutritional features than feather meal (Grazziotin et al., 2006) and could substitute up to 20% of soybean protein when supplemented with methionine in the animal diets (Grazziotin et al., 2008). Moreover, protein hydrolysates obtained from submerged cultivation of keratinolytic bacteria on poultry feathers showed upgraded nutritional value of feather keratin (Grazziotin et al., 2006; Bertsch and Coello, 2006). In a previous study, weights of rats fed with a diet containing enzymatic hydrolysate of horn and hoof (EHHH) from cow and buffalo did not show differences from those of standard rats (Ohba et al., 2003).

Interestingly, the administration of FPH in the food of rats significantly decreased TBARS levels in their organs at levels lower than those of the control rats. This indicates the beneficial effect of FPH against the oxidative stress and confirms its *in vitro* antioxidant activity. This protective effect was also demonstrated with EHHH from cow and buffalo (Ohba et al., 2003).

The antioxidant activity of FPH was associated with the presence of amino acids and small peptides that exert physiological benefits when consumed *in vivo*. According to the literature, the antioxidant peptides have a size lower than 20 amino acids. They are inactive within the sequence of the parent proteins and may be released by proteolytic hydrolysis using commercially available enzymes or proteolytic microorganisms and fermentation methods (Vercruysse et al., 2005). After digestion, bioactive peptides can be absorbed in the intestine and enter the blood stream directly, which ensures their *in vivo* bioavailability and a physiological effect at the target site (Erdmann et al., 2008).

The high rate of TBARS, due to the addition of FM or the soya in the food of rats, is associated with an increase of the enzymatic antioxidant activities such as catalase, superoxide dismutase and glutathione peroxidase. The important expression of these enzymes confirms the oxidative effect of FM and soya. In fact, oxidative stress is known to induce the production of ROS scavenging enzymes (Niskanen et al., 1995). The oxidative stress resulting from the supplementation of the standard food by 5% soya, can be explained by a nutritional imbalance caused by an excess of proteins. Previous studies demonstrated that excess of protein intake can cause an oxidative damage (Youngman, 1993). In the case of diet supplemented by 2.5% of FM, the resulting oxidative stress may be explained by the fact that keratin is indigestible. Nevertheless, when FPH was added, a significant decrease in the antioxidant enzymatic system of various tissues was observed. In fact, FPH compounds protect these tissues from lipid peroxidation by their antioxidant ability and consequently lipid peroxidation is reduced. Dietary supplementation with FPH would allow protecting the animal against oxidative stress.

ACKNOWLEDGMENT

Our thanks are due to Dr. Khaled Jebahi (Higher Institute of Applied Studies in Humanities, Medenine, Tunisia) for his kind help with English.

REFERENCES

Aebi H (1974). Catalase. In: Bergmeyer HU (ed.) Methods of Enzymatic Analysis. Academic Press, New York, pp. 673-684.

Antolovich M, Prenzler PD, Patsalides E, McDonald S, Robards K (2002). Methods for testing antioxidant activity. Analyst, 127: 183-198.
Asada K, Takahashi M, Nagate M (1974). Assay and inhibitors of spinach superoxide dismutase. Agric. Biol. Chem. 38: 471-473.
Ashok B, Ali R (1999). The aging paradox: free radical theory of aging. Exp. Gerontol. 34: 293-303.
Bertsch A, Coello N (2006). A biotechnological process for treatment and recycling poultry feathers as a feed ingredient. Bioresour. Technol. 96: 1703-1708.
Bishop CD, Angus RA, Watts SA (1995). The use of feather meal as a replacement for fish meal in the diet of *Oreochromis niloticus* fry. Bioresour. Technol. 54: 291-295.
Bohne VJB, Lundebye AK, Hamre K (2008). Accumulation and depuration of the synthetic antioxidant ethoxyquin in the muscle of Atlantic salmon (*Salmo salar L*.). Food Chem. Toxicol. 46: 1834-1843.
Chan KM, Decker EA (1994). Endogenous skeletal muscle antioxidants. Crit. Rev. Food Sci. Nutr. 34: 403-426.
Chew BP (1996). Importance of antioxidant vitamins in immunity and health in animals. Anim. Feed Sci. Tech. 59:103-114.
Coward-Kelly G, Chang VS, Agbogbo FK, Holtzapple MT (2006). Lime treatment of keratinous materials for the generation of highly digestible animal feed: 1. Chicken feathers. Bioresour. Technol. 97: 1337-1343.
Dalev PG (1994). Utilization of waste feathers from poultry slaughter for production of protein concentration. Bioresour. Technol. 48: 265-267.
Dastager GS, Chan Lee J, Wen-Jun L, Chang-Jin K, Dayanand A (2009). Production, characterization and application of keratinase from *Streptomyces gulbargensis*. Bioresour. Technol. 100: 1868-1871.
Deshpande SS, Deshpande US, Salunkhe DK (1995). Nutritional and health aspects of food antioxidants. In: Madhavi DL, Deshpande SS, Salunkhe DK (eds.) Food antioxidants. Technological, toxicological, and health perspectives, NY, Basel, Hong Kong: Marcel Dekker, Inc, pp. 361-469.
Dimitrios B (2006). Sources of natural phenolic antioxidants. Trends Foods Sci. Tech. 17: 505-512.
Dinis TC, Maderia VM, Almeida LM (1994). Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch. Biochem. Biophys. 315: 161-169.
Ellman GL (1959). Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82: 70-77.
Erdmann K, Cheung WY, Schröder H (2008). The possible roles of fodd-derived bioactive peptides in reducing the risk of cardiovascular diseases. J. Nutr. Biochem. 19: 643-654.
Fakhfakh N, Kanoun S, Manni L, Nasri M (2009). Production and biochemical and molecular characterization of a keratinolytic serine protease from a chicken feather-degrading *Bacillus licheniformis* RPk. Can. J. Microbiol. 55: 427-436.
Fakhfakh-Zouari N, Hmidet N, Haddar A, Kanoun S, Nasri M (2010). A novel serine metallokeratinase from a newly isolated *Bacillus pumilus* A1 grown on chicken feather meal: biochemical and molecular characterization. Appl. Biochem. Biotech. 162: 329-344.
Farombi EO, Hansen M, Ravn-Haren G, Møller P, Dragsted LO (2004). Commonly consumed and naturally occurring dietary substances affect biomarkers of oxidative stress and DNA damage in healthy rats. Food Chem. Toxicol. 42: 1315-1322.
Flohe L, Gunzler WA (1984). Assay of glutathion peroxydase. Method. Enzymol. 105: 114-121.
Garret HE (1956). Elementary statistics. Now York. Longmans Green and Company, p. 94.
Grazziotin A, Pimentel FA, De Jong EV, Brandelli A (2006). Nutritional improvement of feather protein by treatment with microbial keratinase. Anim. Feed Sci. Tech. 126: 135-144.
Grazziotin A, Pimentel FA, De Jong EV, Brandelli A (2008). Poultry feather hydrolysate as a protein source for growing rats. Braz. J. Vet. Res. Anim. Sci. 45: 61-67.
Halliwell B, Cross CE, Gutteridge JMC (1992). Free radicals, antioxidants and human disease: where are we now? J. Lab. Clin. Med. 119: 598-620.
Hamre K, Kolås K, Sandnes K (2010). Protection of fish feed, made directly from marine raw materials, with natural antioxidants. Food Chem. 119: 270-278.
Huang D, Ou B, Prior RL (2005). The chemistry behind antioxidant capacity. J. Agric. Food Chem. 53: 1841-1856.
Je JY, Qian ZJ, Kim SK (2007). Antioxidant peptide isolated from muscle protein of bullfrog, *Rana catesbeiana Shaw*. J. Med. Food 10: 401-407.
Korycka-Dahl MB, Richardson T (1978). Activated oxygen species and oxidation of food constituents. Crit. Rev. Food Sci. Nutr. 10: 209-241.
Kumar R, Balaji S, Uma TS, Mandal AB, Sehgal PK (2010). Optimization of influential parameters for extracellular keratinase production by *Bacillus subtilis* (MTCC9102) in solid state fermentation using horn meal-A biowaste management. App. Biochem. Biotechnol. 160: 30-39.
Lee J, Kim H, Kim J, Jang Y (2002). Antioxidant property of an ethanol extract of the stem of *Opuntia ficus-indica var. Saboten*. J. Agric. Food Chem. 50: 6490-6496.
Lowry OH, Rosebrough Nira J, Farr AL, Randall Rose J (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265.
Mielnik MB, Aaby K, Skrede G (2003). Commercial antioxidants control lipid oxidation in mechanically deboned turkey meat. Meat Sci. 65: 147-1155.
Miller JH (1972). Experiments in molecular genetics. Cold spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 431-435.
Moure A, Cruz JM, Franco D, Domínguez JM, Sineiro J, Domínguez H, Nunez MJ, Parajo JC (2001). Natural antioxidants from residual sources. Food Chem. 72: 145-171.
Niskanen LK, Salonen JT, Nyyssonen K, Uisitupa MIJ (1995). Plasma lipid peroxidation and hyperglycemia: a connection through hyperinsulinemia? Diabetic Med. 12: 802-808.
Ohba R, Deguchi T, Kishikawa M, Morimura S, Suzuki I (2003). Antioxidative effect of enzymatic hydrolysate of horn and hoof in Rat. Food Sci. Technol. Res. 9: 152-154.
Ouled Haddar H, Zaghloul TI, Saeed HM (2009). Biodegradation of native feather keratin by *Bacillus subtilis* recombinant strains. Biodegradation, 20:687-694.
Papadopoulos MC, El Boushy AR, Roodbeen AE, Ketelaars EH (1986). Effects of processing time and moisture content on amino acid composition and nitrogen characteristics of feather meal. Anim. Feed Sci. Technol. 14: 279-290.
Pasko P, Barton H, Zagrodzki P, Izewska A, Krosniak M, Gawlik M, Gawlik M, Gorinstein S (2010). Effect of diet supplemented with quinoa seeds on oxidative status in plasma and selected tissues of high fructose-fed rats. Plant Food Hum. Nutr. 65: 146-151.
Sahnoun Z, Jammoussi K, Mounir-Zeghal K (1997). Radicaux libres et anti-oxydants : physiologie, pathologie humaine et aspects thérapeutiques = Free radicals: fundamental notions and methods of exploration. Thérapie, 52: 251-270.
Saiga A, Tanabe S, Nishimura T (2003). Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. J. Agric. Food Chem. 51: 3661-3667.
Shih JCH (1993). Recent development in poultry waste digestion and feather utilization- A review. Poult. Sci. 72: 1617-1620.
Vercruysse L, Van Camp J, Smagghie G (2005). ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: A review. J. Agric. Food Chem. 53: 8106-8115.
Yagi K (1976). A simple fluorometric assay for lipoperoxide in blood plasma. Biochem. Med. 15: 212-216.
Youngman LD (1993). Protein restriction (PR) and calorie restriction (CR) compared: Effects on DNA damage, carcinogensis, and oxidative damage. Mutat. Res. 295: 165-179.