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ORIGINAL PAPER

Antioxidant Properties of Fermented Soy during Shelf Life

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Abstract

Glycine max (soybean) is a fundamental food in human nutrition, largely utilized by the consumers, and in particular, fermented soy is mainly used. However, health benefits of the products can change during the shelf life as oxidation processes occur determining alterations of protein and lipid constituents leading to a decrease of nutritional quality. Therefore, the oxidative stability of the fermented soy during the shelf life was studied. The antioxidant potential of this product was evaluated by estimating total phenols, free radical scavenger activity using DPPH and ABTS tests, and the degree of lipid peroxidation, from I up to IX weeks. The antioxidant capacity after an initial decrease, increased again at VII-IX weeks. Lipid peroxidation was evaluated by comparing non fermented and fermented soy. The results disclosed a low amount of peroxides in the fermented soy, suggesting that fermentation brings to an improvement of the product associated to a decreased lipid peroxidation at longer times. Fractions of aqueous extract, obtained at the end of the shelf life from fermented soy, showed an enrichment in antioxidant peptides.

Keywords Shelf life · Fermented soy · Antioxidants · Lipid peroxidation

Abbreviations

ABTS	2, 2′-azinobis(3-ethylbenzothiazoline 6-sulfonate)
Acn	Acetonitrile
BHT	Butylated hydroxytoluene
CHP	Cumene hydroperoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
DTT	Dithiothreitol
GAE	Gallic acid equivalents
FS	Fermented soy
MDA	Malondialdehyde
NFS	Non fermented soy
OPA	*Ortho*-phthalaldehyde
RT	Room temperature
TFA	Trifluoroacetic acid
TPC	Total phenolic compounds
TEAC	Trolox C equivalent antioxidant capacity

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Introduction

Over the last decades, the research on bioactive compounds increased substantially and many studies revealed that various type of food matrix such as berries, milk, quinoa seeds, cereals, canary seeds and soy are enriched in several molecules that can exert a beneficial role on human health [1–6]. In particular, the studies on soy food and supplements have grown considerably and, in most countries, there is broad social acceptance of their health benefits. These products are largely utilized by the consumers, especially by vegetarian people [7]. Soy-based foods are also used by lactose intolerant subjects or by people with allergy to milk proteins [8]. Soybean (*Glycine max*) is a rich source of plant proteins, complex carbohydrates, polyunsaturated fat, soluble fibers and isoflavones [9]. The soy-based food mostly used worldwide is soymilk that is obtained by soaking and grinding whole soybean. Moreover, in Asian countries many foods are prepared with fermented soybean, for example miso, tempeh and natto. In fact, fermentation is a process largely used for food preservation. For this purpose, specific *genera* of lactic acid

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bacteria are usually utilized, such as *Lactobacillus, Streptococcus* and *Leuconostoc* [10]. This approach leads to new products with better properties and different components, such as bioactive peptides. Another outcome of fermentation is the reduction of unwanted constituents present in raw materials, such as phytic acid or allergens [11]. Fermented foods are considered for their capacity, not only to prevent their alterations and to extend shelf life, but especially for their potential benefits on human health [12]. The advantage of this process is to improve the quality of the original product, such as flavour, aroma and appearance. Fermentation is also used to favour the hydrolysis of oligosaccharides and proteins present in plants, that are not digested by the human gut [11, 13]. Moreover, the action of fermented bacteria on soy proteins can lead to release of antioxidant bioactive peptides, bearing beneficial properties both for the maintenance of food quality and for human health [14–18]. While an extensive literature can be found about the effects of soy foods with antioxidant properties, regarding the fermented soy-based beverage there is still much to be investigated [19].

In this paper, samples of fermented soy were analysed during the whole shelf life, and for further 3 weeks, in order to examine the antioxidant properties of the product [20]. In order to determine the antioxidant capacity during the shelf life, different assays (total phenolic content, ABTS and DPPH scavenging assay and lipid peroxidation) were performed weekly. Then, peptide fractions from fermented soymilk were extracted to evaluate if the potential antioxidant effects can be referred to bioactive peptides.

Materials and Methods

The material and methods section is presented as Online resource.

Results and Discussion

The studies on fermented soy properties were carried out at the indicated weeks (I to IX) until the end of the shelf life and, in addition, up to 20 days after the expiration date. To this purpose, different tests were employed to estimate total phenolic groups and the antioxidant properties through ABTS, DPPH scavenging assays and MDA formation. In addition, protein fragmentation was also evaluated. Furthermore, the extraction of peptide fractions was performed in order to obtain samples enriched in bioactive peptides.

Determination of Total Phenolic Content

Preliminary evaluation of the principal phenolic compounds (especially isoflavones and tocopherols) present in the non fermented and fermented soy are reported in Table S2 (Online resource). The phenolic components of soybean are found mainly as beta-glucoside conjugates as indicated by Zhao and Shah [21] and, after the fermentation process according to the literature [21] a significant increase of isoflavones was observe.

Therefore, the total phenolic compounds concentration (TPC) was estimated during all the shelf life (Fig. 1 a). Tests were repeated once a week as indicated and the values are reported as micrograms of gallic acid equivalents (GAE) *per* 100 mL of sample. First, these results assess that, during the

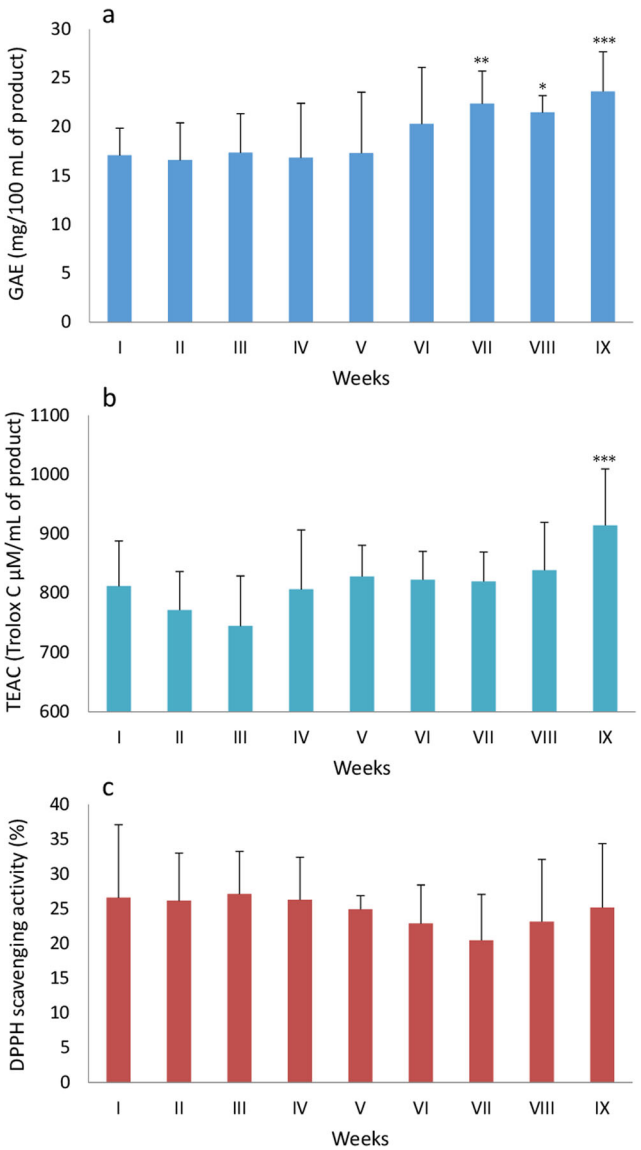

Fig. 1 Total phenolic content and antioxidant activity with ABTS and DPPH assays in fermented soy. Fermented soy (1 mL of aqueous extract) was treated with Folin-Ciocalteau reagent as described in Material and methods section. The absorbance was recorded at 750 nm (**a**). The antioxidant capacity of the aqueous extract (0.02 mL) was estimated using the ABTS test **(b)** and the DPPH scavenging activity **(c)**. Means of 5 experiments were compared with the first week values (3 replicates for each experiment) (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$)

shelf life, the TPC content of the products was not significantly altered. As shown in Fig. 1 a, the total phenolic content is stable for 4-5 weeks, although an increase of the phenolic compounds starting from the sixth week of shelf life is apparent.

This observation is confirmed using other tests able to validate the antioxidant power such as ABTS (see below).

Evaluation of Antioxidant Activity with the ABTS and DPPH Scavenging Assay

As shown in Fig. 1 b, the antioxidant capacity of the aqueous extract (0.02 mL) of fermented soy was tested using the ABTS test. First, a calibration standard curve with Trolox C was set up and the data were expressed as equivalents of Trolox C (TEAC). During the shelf life, substantial differences were observed, and, as shown in Fig. 1 b, antioxidant activity in fermented soy slightly decreased during the first three weeks, where the antioxidant capacity ranges from 811.72 TEAC (I week) to 744.33 TEAC (III week). Subsequently, the antioxidant activity was stable until the seventh week, but increased markedly at the end of the shelf life (914.36 TEAC). Therefore, we can verify that the product is rather stable during the shelf life and, at the end of this period, the antioxidant properties of the fermented soy increase. Furthermore, DPPH scavenging assay was used to validate the previous results. As reported in Fig. 1 c, antioxidant activity in fermented soy was stable during the first four weeks, while for longer times the antioxidant activity slightly decreased until the seventh week. However, at the end of the shelf life, the antioxidant activity exhibited values similar to those observed in the first week. On the whole, these results, obtained with three different methods, point out that fermented soy appears stable during the shelf life, while at the end of this period, the antioxidant properties of the fermented soy increase.

Lipid Peroxidation during the Shelf Life as Marker of Antioxidant Capacity

In order to further assess the antioxidant properties during the shelf life and for the following three weeks, the alterations of lipid components were considered by evaluating malondialdehyde (MDA), a lipid peroxidation product. In these experiments, a comparison between soy fermented milk and soymilk (*i.e.*, before the fermentation process) was performed.

Samples (0.1 mL) of non fermented (NFS) and fermented soy (FS) were tested for MDA production as described in the Material and methods section. MDA formation was followed in four different lots of each product from I to IX week. As apparent in Fig. 2 a, the products did not undergo major oxidative alterations during the entire shelf life. Furthermore, the fermented product was less sensitive to lipid peroxidation

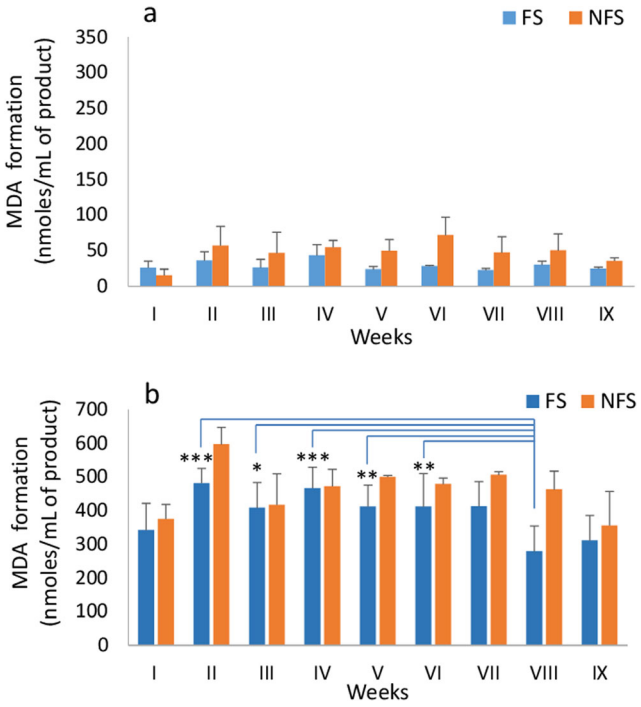

Fig. 2 Lipid peroxidation in non fermented (NFS) and fermented soy (FS) in basal conditions (**a**) and after stimulation in the presence of CHP/hemin (**b**). MDA formation was determined on samples of fermented or non fermented soy, by following the formation of the fluorescent adduct with thiobarbituric acid Ex, 530 nm; Em, 540 nm). Means of 5 experiments were reported (3 replicates for each experiment) (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$)

compared to the non fermented sample. However, the MDA values are higher if compared with similar experiments obtained with products derived from cow milk such as yogurt [22], as soy been contains a higher content of potentially peroxidizable unsaturated fatty acids. In addition, lipid peroxidation was also induced in the presence of cumene hydroperoxide (CHP) and hemin (Fig. 2b), well known inducers of lipid peroxidation. In fact, heme compounds can mediate the decomposition of hydroperoxides such as CHP or lipid hydroperoxides generating free radicals, that stimulate lipid peroxidation in biological samples [23, 24]. Therefore, in this condition, a strong increase in MDA formation took place for both products. Once again, the fermented product is less prone to undergo lipid peroxidation and a biphasic effect on MDA production was detected. As reported in Fig. 2 b, in fermented soy MDA gradually incremented until the IV week of shelf life, and then, a progressive decrease was observed. This occurrence probably depends on the activity of bacteria present in the fermented soy resulting in the production of bioactive peptides with potential antioxidant properties [15]. This trend is in accordance with the observed increase of antioxidant activity at the end of the shelf life indicating that the fermentation process is able to give a protection against oxidative stress.

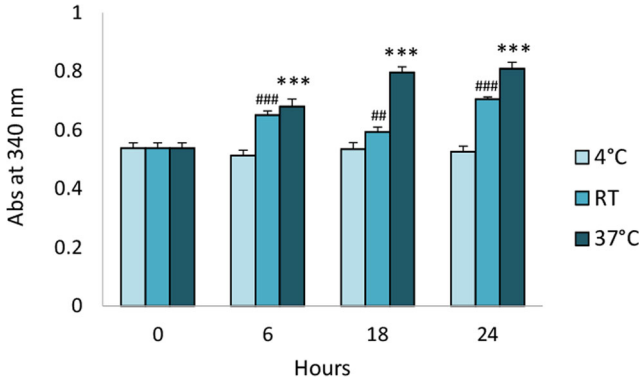

Fig. 3 Proteolytic fragmentation of the samples was followed at 0, 6, 18 and 24 h using the o-phthalaldehyde test, in fermented soy treated in three different storage conditions: 4 °C, RT and 37 °C. Means of 3 experiments were compared with the values at 0 time (3 replicates for each experiment) (*** and $^{###}$ $p < 0.001$, ** and $^{##}$ $p < 0.01$)

Evaluation of Protein Breakdown with o-Phthalaldehyde Assay

Another parameter investigated for fermented soy was its capability to undergo a proteolytic process. To this purpose, samples (VIII weeks old) stored at different temperature for 24 h were tested using the o-phthalaldehyde assay, following the absorbance increase at 340 nm. As shown in Fig. 3, samples maintained at 4 °C were resistant to proteolysis, while, an increase of the proteolytic process during the 24 h is apparent when the fermented soy products were stored at RT. In addition, fermented soy samples stored at 37 °C, showed a high proteolysis, probably due to the re-activation of the present microorganisms. This result also indicates that, at the end of the shelf life, the fermented bacteria are still alive in the food matrix and can act on proteins and other molecules, actively releasing bioactive peptides and other antioxidant compounds.

Extraction and Analysis of Peptide Fractions

As reported in Fig. 1, at the end of the shelf life, an increase of the antioxidant capacity of fermented soy products was observed. Furthermore, the proteolytic activity of fermented bacteria was detected with the o-phthalaldehyde assay (Fig. 3), indicating that the microorganisms were still alive at the VIII week of shelf life. These results suggested that antioxidant bioactive peptides could be released from soy proteins due to the proteolytic action of the fermented starter cultures. To evaluate the presence of these peptides ameliorating the shelf life of the product, aliquots of the aqueous extract, obtained at the VIII week of the shelf life, were subjected to chromatographic separation on STRATA column, as reported in the Material and methods section.

First, to assess the enrichment in peptides, a determination of protein content was carried out. Therefore, as reported in Fig. 4 a, aliquots of lyophilized aqueous extract (lanes **a** and **b**, 30 and 60 μg, respectively) and of lyophilized 30% Acn fraction (lanes **c** and **d**, 30 and 60 μg, respectively) were subjected to Tris/Tricine SDS-PAGE to detect the low molecular weight proteins. A net increment of protein content was apparent in the lanes **c** and **d** compared to lanes **a** and **b,** indicating that, the extraction with STRATA column was a crucial selective process. Subsequently, the exact content of proteins was estimated using Bradford assay. As reported in Fig. 4 b there was a 5.79 times enrichment in proteins.

Antioxidant Properties of the Enriched Peptide Fractions

The fraction eluted with 30% Acn, was studied for its antioxidant properties and tested with ABTS and DPPH scavenging assays. Therefore, a comparison between 30% Acn fraction and the whole aqueous extract, using the lyophilized products, was carried out. The analysis of the two samples showed that

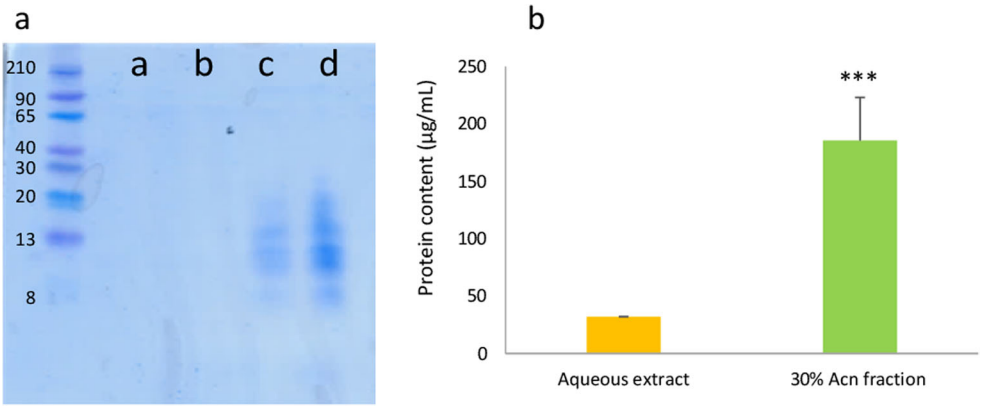

Fig. 4 Comparison of protein content between aqueous extract of soy fermented milk and 30% Acn fraction. **a**: Tris/Tricine SDS-Page: lanes (**a**) 30 μg, (**b**) 60 μg of aqueous extract and (**c**) 30 μg (**d**) 60 μg of 30% Acn fraction. **b**: Aqueous extract and 30% Acn fraction were estimated for the protein content using the Bradford assay. Means of 3 experiments were reported (*** $p < 0.001$)

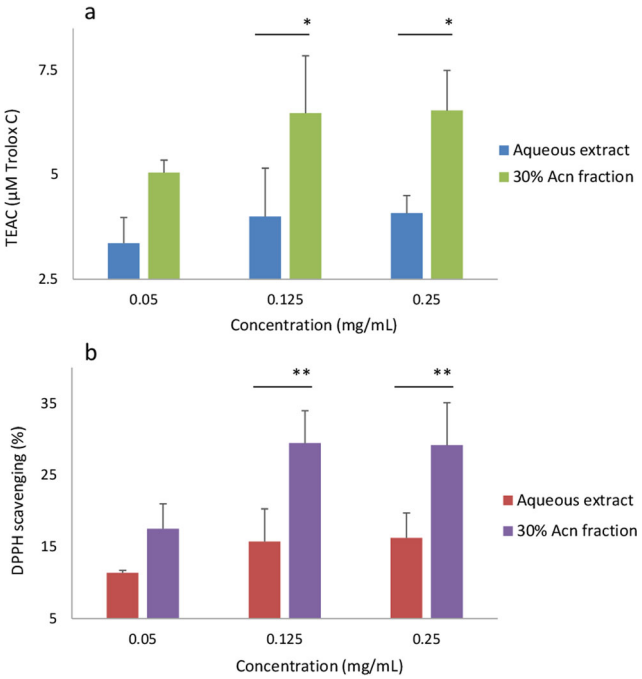

Fig. 5 Antioxidant activity of fermented soy aqueous extract and 30% Acn peptide fraction with ABTS (**a**) and with DPPH (**b**) assays. Increasing concentrations of fermented soy aqueous extract and 30% Acn peptide fraction were evaluated. Means of 3 experiments (8 replicates for each experiment were performed) (** $p < 0.01$, * $p < 0.05$)

the peptide-enriched fraction exhibited a large increase of antioxidant capacity. In particular, TEAC units were 4.07 for the aqueous extract (referred to 0.25 mg/mL), while 30% Acn fraction reached 6.54 TEAC units (Fig. 5a). In addition, DPPH scavenging for Acn fraction (0.05 mg/mL) increased of about 1.8 times compared to aqueous extract (Fig. 5 b). All these results support the fact that many antioxidant peptides are present in the 30% Acn extract.

Conclusions

In our data, fermented soy product showed a large stability during all shelf life, associated to an increase of antioxidant power at the end of the shelf life, possibly due to the formation of bioactive peptides. Moreover, lipid peroxidation estimation is in accordance with these results, because at the end of shelf life, both in basal conditions as after stimulation with CHP/hemin, a decrease of MDA formation was observed. In fact, the action of fermented microorganism after VI-VIII weeks, could determine the production of a large number of small peptides with antioxidant properties. In this regard, food fermentation inhibits the growth of other microorganism in a process dependent on the production of specific metabolites from fermenting culture able to prevent the colonization of food matrix [25]. In addition, it is well known that fermentation has the potential to improve the bioaccessibility and the bioactivity of polyphenol compounds [21]. Moreover, the release of antioxidant compounds by the action of fermenting bacteria can increase the shelf life by inhibiting the redox reactions occurring during food spoilage. In this soy fermented study, we assume that the peptide fraction has a crucial role in imparting antioxidant properties. In fact, many peptides are released during the fermentation process as previously reported by Roblet et al. [26].

Furthermore, research devoted to the isolation and determination of the sequences of peptides endowed with antioxidant properties will be of great interest in defining the specific properties of the peptide molecules involved in the protective effect.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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