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Proteomic/peptidomic profile and *Escherichia coli* growth inhibitory effect of *in vitro* digested soya protein

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ABSTRACT

Plant proteins contain bioactive peptides with functional properties and physiological activities. In the present work, the bioactive peptides produced during *in vitro* gastrointestinal digestion of soya protein isolate were investigated. Protein samples were subjected to simulated gastrointestinal digestion with a generation of permeate (<3 kDa) and retentate (>3 kDa) fractions. The permeate was analysed by nano-liquid chromatography electrospray ionisation tandem mass spectrometry (LC-nano ESI MS/MS) using a shotgun peptidomic approach, and the retentate was further digested with trypsin and analysed using a shotgun proteomic approach. Based on protein profile observed, the retentate was further tested for its potential antimicrobial activity by evaluating the inhibitory effect on *E. coli* growth. In the present study the peptidomic/proteomic characterisation of permeate and retentate fractions revealed the presence of bioactive peptides and proteins associated with antioxidant, ACE-inhibitory, anti-hypertensive and antimicrobial activities. The presence of potentially antimicrobial proteins in the retentate fraction is supported by a marked *E. coli* F18+ growth inhibitory activity of the same fraction. In particular, the growth inhibitory effect was significant from one until six hours of incubation with 0.65–2.6 mg/ml of *in vitro* digested soya. The obtained data confirmed that soya-based supplements may have potential beneficial effects after human consumption, and they may be re-cycled for animal nutrition in line with the circular economy concept.

HIGHLIGHTS

- Soya-based supplements are relevant sources of bioactive molecules.
- Soya based supplements could be re-cycled for animal feeding considering the circular economy concept.

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Introduction

A growing body of scientific evidence has revealed that many food proteins and peptides exhibit specific biological activities in addition to their established nutritional value (Fekete et al. 2016; 2018). Besides the well-demonstrated functional activity of milk peptides derived from the digestion of proteins (Giromini et al. 2019a; Giromini et al. 2019b; Politis and Theodorou, 2016), several plant proteins have also demonstrated that they contain bioactive molecules beneficial for the health (Giromini et al. 2017). However, although alternative plant products are considered potential sources of bioactive peptides, soya and derivative products are the main matrices in which the Angiotensin-Converting Enzyme (ACE)-inhibitor bioactivity was described (Piovesana et al. 2018). Many bioactive peptides have already been isolated from soya proteins and previous studies have demonstrated their potential functional properties and physiological activities, such as antihypertensive, hypocholesterolaemic, antioxidant, and anticancer (Wang and De Mejia 2005). Soya based supplements are largely used in

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sports nutrition because of their high quality protein composition and represent one of the most valid alternative to milk protein (mostly whey) in case of allergy. However, few data are available on the characteristics of the bioactive peptides and proteins in soya, in particular after simulated digestion. Moreover, soya at present is the main protein source administered in feed for farm animals; it provides isoflavone which is known to promote tissue growth in piglets and to prevent diseases. In fact, although in the last decade the animal feed research has been focussed on alternative protein sources in particular in those regions not conducive for soya production, the animal full growth and productive performances cannot be achieved without traditional protein sources administration (such as soya proteins) (Nahashon and Kilonzo-Nthenge 2011). Peptidomics and proteomics approaches allow to track the release of bioactive peptides during food digestion. In fact, gastro-intestinal digestion of dietary proteins is a crucial step for the release of encrypted peptides with potential biological activity. The release of bioactive peptides from different food sources has been followed in a number of studies and peptidomics analysis was employed to study the differences in protein hydrolysis and bioactive peptide release after in vitro gastro-intestinal digestion of dairy and plant products (Capriotti et al. 2015; Martini et al. 2021). ; Our previous experiments (Giromini et al. 2017; 2019a) demonstrating relevant ACE-inhibitory activity and antioxidant capacity in dairy and soya protein isolate after *in vitro* digestion, although lower than those observed in milk proteins, stimulated further investigation of soya peptides generation after digestion. Therefore, the objective of this study was to investigate the bioactive peptides and protein profile generated by simulated gastrointestinal digestion of soya protein using a shotgun proteomic/peptidomic approach. The retentate fraction of the digesta, which may represent the fraction able to reach the microbial population of the large intestine, was also tested for its potential antimicrobial activity by evaluating its inhibitory effect on *E. coli* growth.

Materials and methods

In vitro *digestion*

The soya sample tested in the present study was soy protein isolate (MyProtein, Northwich, UK) containing 90 g/100 g of powder.

In vitro digestion was performed according to the method of Minekus et al. (2014) and further adapted by our group (Giromini et al. 2017; 2019a). Briefly, the digestion procedure involved three phases. For the oral phase α-amylase in 1 mM $CaCl_2$, pH 7 was added to the samples and they were incubated for 30 min at 37 °C on a shaker. For the gastric phase, the pH was decreased to 2 with 6 M HCl and pepsin in 0.1 M HCl was added. The samples were then incubated for 120 min at 37 °C on a shaker. For the small intestinal phase, the pH was increased to 7 with 6 M NaOH and a mixture of pancreatin and bile in $NaHCO_3$ 0.5 M was added to the soya digest before the final incubation of 180 min at 37 °C on a shaker was performed. A positive control (whey isolate protein with recognised level of digestibility of 96% v/v) and negative control (enzyme mixture without protein sample) were included as reference protein samples and for stability tests in all digestions performed ($n = 3$). At the end of the digestion, the total digesta obtained was transferred to 3 kDa cut-off membrane (Vivaspin 20 Sartorius), previously activated with a solution with 0.1% BSA. Samples were centrifuged for 20 min at 3500 x g to obtain permeate (simulation of potentially absorbed peptides and polypeptides <3 kDa) and retentate (simulation of peptide and polypeptides $>$ 3 kDa which can potentially reach the large intestine) fractions. Aliquots from permeate and retentate fractions were sampled and snap frozen in liquid nitrogen to stop enzyme activity before storing at −80 °C for further experiments.

Proteomic and peptidomic profile

Permeate and retentate samples were analysed by LC-nano ESI tandem mass spectroscopy using a shotgun-label free approach to identify peptides and proteins. The permeate was analysed by MS/MS to identify endogenous peptides present in the samples, the so called peptidomic strategy while the retentate was further digested using sequence-grade trypsin with a protein:protease ratio 20:1 (Tedeschi et al. 2011) with a proteomic strategy (Figure 1). The digestion with trypsin is a normal procedure in the proteomic MS/MS analysis in order to identify the proteins based on the sequence of the corresponding tryptic peptides. Trypsin generates experimentally observable peptides that can be used to uniquely identify a protein (Vernocchi et al. 2014). Mass analysis was carried out as described in Aletti et al. (2016) for the peptidomic strategy and in Maffioli et al. (2018) for the proteomic analysis.

In details, for the peptidomic strategy the permeate was analysed without previous proteolysis by trypsin and residual proteins eventually still present upon filtration were further precipitated with two volumes of

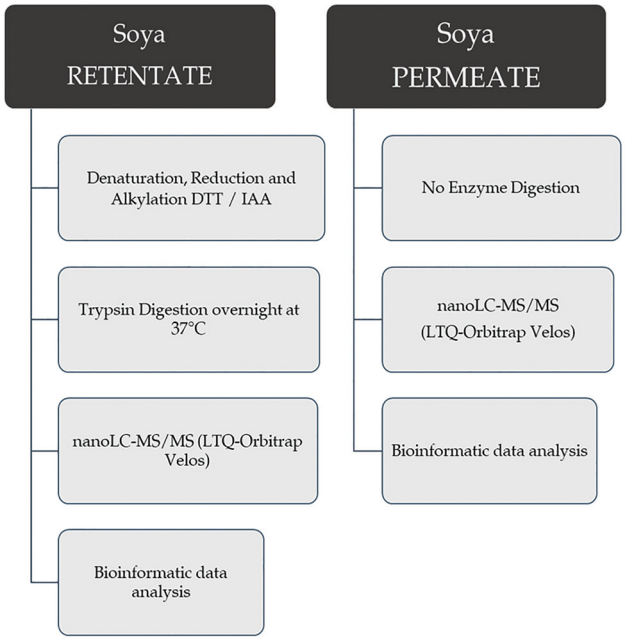

Figure 1. Peptidomic and proteomic workflow of soya protein sample.

cold acetonitrile containing 0.1% of trifluoroacetic acid and centrifuged before LC-ESI-MS/MS analysis. The supernatant was then dried (Speed Vacuum), dissolved in 1% (v/v) formic acid and desalted by Zip-Tip C18 (Millipore) before nanoLCESI MS/MS analysis.

For the proteomic strategy, upon reduction, derivatization and trypsin digestion, the retentate was desalted by Zip-Tip C18 (Millipore) and analysed by mass spectrometry.

NanoLCESI was carried out using a LTQ Orbitrap Velos (Thermo Fisher Scientific) equipped with a Dionex UltiMate 3000HPLC System and a Proxeon nanoelectrospray ion source (Thermo Fisher Scientific). Data acquisition was controlled by Xcalibur 2.0 and Tune 2.4 software.

Peptidomic and proteomic data analysis

MS spectra were searched against the NCBI sequence database (release 24.01.2017) by the Sequest search engine contained in the Proteome Discoverer 1.4.0 software (Thermo Fisher Scientific Inc., USA). The following parameters were used: 10 mg/kg for MS and 0.5 Da for MS/MS tolerance, Met oxidation, N-terminal acetylation and Gln/Asn deamidation as variable modifications. Carbamidomethylation of Cys as fixed modification and trypsin (2 misses) as protease were parameters further added in the proteomic analysis. Only peptides with False Discovery Rate 1% (against decoy) and Xcorr 1.5 were included for positive. Two replicates were carried out for each sample in the MS analysis. To find potentially bioactive peptides with an in silico approach, all peptides in the permeate identified by peptidomics were searched in SATPdb (Singh et al. 2016) and BIOPEP-UWM database (Minkiewicz et al. 2008), two databases of structurally annotated therapeutic peptides, that integrated different peptide databases having diverse nature of peptides. SATPdb (http://crdd.osdd.net/raghava/satpdb/) is a database of structurally annotated therapeutic peptides, curated from 22 public domain peptide databases/datasets. In the two databanks the peptides are classified based on their major function, therapeutic property and subfunction. By conducting the analysis on these databases we searched for exact matches also considering possible further proteolysis, thus making it possible to identify some potentially bioactive peptides. In particular, in order to consider possible further proteolysis, the search was performed in Excell keeping a minimum sequence length of 6 amino acids and applying a "IF" nested function to a matrix which compared the sequence of each peptide found with the ones of the database as reported in Aletti et al. (2016).

E. Coli *growth inhibitory activity*

The antimicrobial activity of *in vitro* digested soya was evaluated on cultures of *E. coli* F18+ as it is one of the most common pathogen of swine GI tract .

The ETEC strain, harbouring F18 (F18+) adhesive fimbriae, was obtained from IZSLER (Brescia, Italy). The bacteria were grown at 37 °C with shaking (150 rpm) in LB broth for 12 h prior to being used as inoculants for all experiments. In particular, overnight-grown *E. coli* F18+ were inoculated into tubes containing LB medium supplemented with 0–2.6 mg/ml of soya retentate or with enzyme mixture used for the in vitro digestion (negative control). The bacterial cultures were adjusted to identical densities by spectrophotometry (600 nm) prior to the inoculation (approximately 10^6 CFU/ml). All tubes were incubated aerobically with shaking (150 rpm) at 37 °C up to 6 hours. The bacterial growth was determined via measurement of optical density of each culture at 600 nm (OD600) at 1 hour interval in a spectrophotometer (UV/VIS Lamba 365, PerkinElmer) (Reggi et al. 2020). All data obtained were log transformed based on a calibration curve, obtained monitoring the *E. coli* growth over time. At regular intervals (every hour), the OD was monitored and, in parallel the colony forming unit (CFU) was estimated by the plate counting method. The research reported is in full compliance with all relevant codes of experimentation and legislation

Table 1. Bioactive peptides identified in the soya permeate fraction by searching in SATPdb (Singh et al. 2016) and BIOPEP-UWM (Minkiewicz et al. 2008) databases and considering possible further proteolysis.

Peptides listed in the Database	Peptides identified in soya permeate, < 3 kDa
ACE- inhibitor activity	
NWGPLV	KYEGNWGPLVNPESQQGSP
NWGPLV	LKYEGNWGPLVNPESQQGSP
NWGPLV	LKYEGNWGPLVNPESQQGSPR
Antioxidant activity	
ALEPDHR	NLNALEPDHRVESEGGL
ALEPDHR	NALEPDHRVESEGGLIQT
Anti-hypertensive activity	
NWGPLV	KYEGNWGPLVNPESQQGSP
NWGPLV	LKYEGNWGPLVNPESQQGSP
NWGPLV	LKYEGNWGPLVNPESQQGSPR

The exact matches with the peptides listed in the Databanks are indicated in bold in the permeate peptides.

Statistical analysis

Statistical analysis of antimicrobial data was performed using GraphPad-Prism 8. In particular, *E. coli* growth data were subjected to analysis of variance using the one-way ANOVA procedure. The differences between means were compared using Tukey's test and considered statistically significant at $p < .05$. Data are presented as least square means ± SEM.

Results

We characterised the peptide and protein panel of soya retentate and permeate generated by *in vitro* digestion using a static digestion protocol, which simulate human and monogastric animal digestion process. This approach provided a suitable strategy to produce and characterise the molecules that can be generated during *in vivo* digestion. Thus, the final hydrolysate obtained represents a pool of peptides and polypeptides resembling those generated during the physiological digestion of soya proteins. Small peptides and polypeptides (<3 kDa) could be absorbed by epithelial cells along the small intestine and enter the blood circulation where they can exert different biological functions.

Permeate was characterised using a shotgun-peptidomic approach while the retentate was further digested with trypsin and analysed with a shotgun-proteomic approach. The complete list of the proteins and peptides identified in the soya retentate and permeate are reported in the Supplementary Tables S1 (permeate) and S2 (retentate), respectively. All the peptidomic data (Table S1) were searched in SATPdb and BIOPEP-UWM databases to find potentially bioactive peptides by an in silico strategy, taking into account possible further proteolysis of the peptides identified in the permeate, due to peptidases potentially present in the gastrointestinal tract.

The antioxidant and ACE inhibitory activities of soya permeate have been previously described by our group (Giromini et al. 2017; 2019a). Specifically, soy proteins, as most of the common food proteins, are a natural source of AOX compounds, which are able to exert remarkable cytoprotective effect (Gao et al. 2019). With regard to ACE inhibitory activity, it has been reported (Giromini et al. 2017) that this property was significantly reduced in soya permeate fractions compared to the undigested and to retentate fraction (>3kDa). These findings demonstrate that digestion may modulate protein bio-activities, such as those related to ACE inhibition. In some instances, although the presence of bioactive peptides is proven by *in silico* analysis, the quantity of such peptides may not be enough to guarantee such activity.

In accordance with those results, peptides with ACE inhibition, antioxidant and anti-hypertensive activity were identified in the permeate (Table 1).

The data presented confirm that previously observed bioactivities (Giromini et al. 2019a) are related to the presence of antioxidant and ACE inhibitory peptides in the digested soya fractions.

Only a few previous studies have assessed the release of bioactive peptides after soya digestion (Capriotti et al. 2015). This work allowed the identification of a greater number of bioactive peptides compared with the data reported in Table 1, probably due to the different digestion conditions applied an on the different type of soya samples digested (soya seed, soya milk vs pure protein). Moreover, there is a lack of information about soya protein bioactivity and the effective release of peptides associated with that bioactivity. Therefore, the present study adds new information by comparing the biological activities of digested proteins with the identification of bioactive peptides and proteins.

The retentate fraction was analysed to identify proteins with known biological activity and the results revealed the

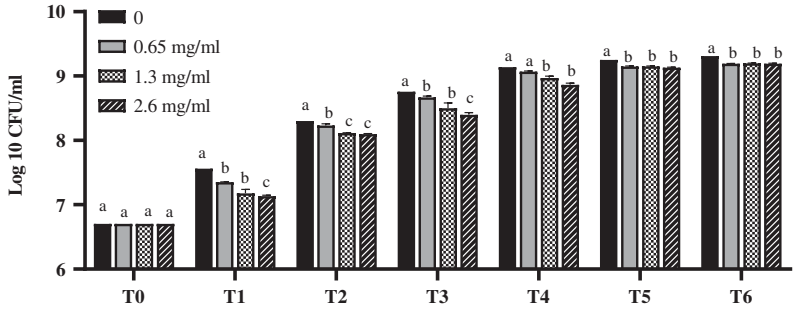

Figure 2. Effect of *in vitro* digested soya protein retentate (0.65–2.6 mg/ml) on E. coli F18+ growth over time (from T0, soya addition, up to T6, six hours). Digestion and antimicrobial analysis were performed in triplicate ($n = 3$). Data are expressed as OD600 least square means ± S.E.M. At each time point, different superscript letters indicate significant difference at $p < .05$. 0 mg/ml represent the *E. coli* F18+ growth over time (CTR, 0 mg/ml). The *in vitro* digested soya stock solution was 0.1 g/ml.

presence of proteins with mainly antimicrobial properties (Table S2). In particular, two uncharacterised proteins (C6SWW4, I1LJD0) were identified that show 99.5% identity with Kunitz-type trypsin inhibitor KTI1 of Glycine max and of the complex porcine pancreatic trypsin soya inhibitor (157838209). Previous studies demonstrated the effect of a serine protease inhibitor on the growth of pathogenic and non-pathogenic microorganisms (Macedo et al. 2016) with both antibacterial and antifungal activities (Dokka and Davuluri 2014). Glycinin (Q9SB11 and P11828) and beta-conglycinin alpha-like protein (I1NGH2) reported in Table S2, are two proteins with an equivalent antimicrobial activity against *E. coli* (Vasconcellos et al. 2014). Furthermore, the uncharacterised protein (I1LKD3) shows 72.6% of identity with the vicilin-like antimicrobial peptides 2–2 of Macadamia integrifolia, with well-known antibacterial and antifungal activities against a range of species (Marcus et al. 1999; Wang et al. 2012).

Overall, the data clearly suggest that some of the proteins identified in the retentate have antimicrobial activity and may represent an excellent natural source for antimicrobial compounds in the gut. To verify this hypothesis, the retentate was tested for the potential antimicrobial activity by evaluating its inhibitory activity on *E. coli* F18+ growth (Figure 2).

As reported in Figure 2, the presence of potentially antimicrobial proteins in the retentate fraction is supported by a marked *E. coli* F18+ growth inhibitory activity of the same fraction at the concentration of 0.65–2.6 mg/ml of retentate. In particular, the growth inhibitory effect was significant ($p < .05$) from 1 hour of incubation (T1) until the end of the experiment (T6) with 0.65–2.6 mg/ml of *in vitro* digested soya, compared with control (0 mg/ml). The concentrations applied in the present study derived from preliminary experiments and can be considered physiologically relevant as only diluted level of soya protein digest can effectively reach the cell epithelium and the bacterial population in physiological condition (Giromini et al. 2019a).

However, it is of note that the *in vivo* digestion process is far more complicated. The presence of exopeptidases and endopeptidases will further degrade these oligopeptides, resulting in release of higher number of bioactive peptides. Thus, further studies *in vitro* and in humans and animals are required for a more detailed understanding of the functional effects of bioactive peptides from soya. Finally, soya protein besides being a source of bioactive peptides for humans, it may also represent a promising font of bioactives for monogastric animals. The re-cycling of proteins supplements with high protein concentration (e.g., >90% protein) as feed ingredients may represent a strategy to meet circular economy principles by also providing animals with high quality proteins which are also a source of functional ingredients. In this respect, this study represents an example of the application of omics technologies (as peptidomics) for the valorisation of a potential agro-food waste for animal nutrition purpose.

Conclusions

In the present study the peptidomic/proteomic characterisation of soya permeate and retentate fractions revealed that permeate is a source of bioactive peptides with antioxidant, ACE-inhibitory and antimicrobial activity, while the retentate fraction showed to be a source of antimicrobial proteins. These results confirmed that soya-based supplements (as for milk-based supplements) have potential beneficial effects in human and animal nutrition, particularly farm animals. This is quite important since among the ingredients used to prepare protein supplements, dairy and plant protein sources are the most commercialised. Moreover, these protein supplements, after the expiration date, cannot be used for human nutrition purposes but they could be re-cycled for animal feeding in light of the circular economy concept.

Ethical approval

All experimental procedures were conducted *in vitro*.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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