Antioxidant activity and identification of food proteins by digestive enzyme supplementation and fermentation with *Lactobacillus kefiri*

Ufuk Gökçe Ayrancı[1], Anıl Şeker[1], Sevda Arısoy[2], Hüseyin Çimen[3],
Özlem Üstün-Aytekin[2]*

[1]*Department of Food Engineering, Faculty of Engineering, Pamukkale University, Kinikli-Denizli, Turkey*

[2]*Department of Nutrition and Dietetic, Faculty of Health Sciences, Health Sciences University, Istanbul, Turkey*

[3]*Department of Genetics and Bioengineering, Yeditepe Proteomics and Mass Spectrometry Laboratory (YediPROT), Yeditepe University, Istanbul, Turkey*

Corresponding author: ozlem.aytekin@sbu.edu.tr

Abstract: Casein, gluten, and soy protein are widely used in food processing for structure, texture, and flavour improvement. These large proteins might be hydrolysed to shorter peptides or amino acids, which provide antioxidant activities through enzymatic and fermentative food processes. Casein, gluten, and soy protein were digested with an enzyme supplement product containing dipeptidyl peptidase IV (DPPIV) and protease in this study. Then, each protein was hydrolysed by *Lactobacillus kefiri* strain. 2,2, diphenyl 1-picryl hydrazyl (DPPH) radical scavenging activity and reducing power (*RP*) were measured for undigested and digested samples. According to our results, all proteins were hydrolysed. Soy protein demonstrated the highest IC_{50} value of DPPH for undigested (2.64 mg/ml) and digested samples (1.56 mg/ml) as well as the highest *RP* value (0.171 for undigested and 0.234 for digested at 700 nm). On the other hand, casein provided the weakest DPPH radical scavenging activity (1.58 ± 0.041% for undigested and 21.86 ± 0.012% for digested samples). A strong correlation was found between cell growth and antioxidant activity of casein during the microbial fermentation. In addition, the changes in protein expression levels by microbial fermentation were analysed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Mass spectrometry-based protein identification studies revealed that EF-G, DNA-K, and DNA-J were mainly overexpressed indicating *L. kefiri* adapts itself by changing the overall proteome.

Keywords: casein; DPPH radical scavenging activity; gluten; *Lactobacillus kefiri*; MALDI-TOF/TOF; soy protein

There are enormous protein resources in food industry such as casein, gluten, and soy protein. These proteins are widely used in food processing for structure, texture, and flavour improvement. However, the lack of solubility of these large proteins, particularly gluten and casein could be problematic. Gluten from wheat consists of two components, which are gliadin and glutenin. Gliadins are single-chain polypeptides with a variety of molecular weight (*MW*) between

30 000 and 80 000 Da, whereas glutenins are multi-chain polypeptides with MW ranging from 80 000 to several million Daltons (Payne 1987; Shewry *et al*. 1992; Wang *et al*. 2006). In the food industry, wheat gluten is used mainly as an additive for enhancement of flour baking quality and is readily available in large amounts at low cost.

Caseins are composed of four major proteins; α_{s1}-casein, α_{s2}-casein, ß-casein, and κ-casein. Their MWs

are between 19 000 and 30 000 Da (McMahon & Oommen 2013). In addition, there are also whey proteins along with other minor proteins. There is a great number of applications for casein in the food industry, particularly dairy product manufacturing, food for infant and baby, soups, sauces and dressings, and meat products.

Soy protein comprises of albumins and globulins. Globulins are dominant storage proteins around 168 000 Da (Utsumi *et al.* 1997). Typical functions for soy proteins are gelation, emulsification, foaming, cohesion-adhesion, elasticity, viscosity, solubility, and water absorption and binding (Nishinari *et al.* 2014; Gaspar & Góes-Favoni 2015).

Proteolytic actions of commercial enzymes, microbial fermentation, heat treatment, pH modification or digestion enzymes lead to the hydrolysis of those large proteins. Therefore, compounds are generated by antioxidant activities (Villegas *et al.* 2014; Li-Chan 2015).

Enzymatic hydrolysis, generally with proteases, has been known to be very effective in improving the functional properties of food proteins. For instance, enzymatic digestion of egg-white protein has been found to increase antioxidant activity (Cho *et al.* 2014). This enhancement can be explained by the hydrolysis of more active amino acid include radical groups (Matoba 2002). In addition to this, their corresponding positioning in peptide sequence plays an important role in the antioxidant activity of peptides (Rajapakse *et al.* 2005).

Microbial fermentation has been used in food industry for years to enrich flavour, well sensory, nutritive or preservative properties via protein hydrolysis with microbial proteases. The proteolytic system of lactic acid bacteria (LAB) is well known and includes intracellular endopeptidase, exopeptidase, and cell membrane-bound proteinase (Üstün & Öngen 2012). Due to these enzymes, some LAB has anti-oxidative activity and can reduce the risk of reactive oxygen species accumulation during food ingestion (Pihlanto 2006). These bacteria may lead to the production of new proteins from casein, gluten, and soy protein.

Kefir is a unique fermented dairy product that is produced by a mixture of lactic acid bacteria and yeast. *Lactobacillus* is the most frequent genus detected in kefir, and particularly *Lactobacillus kefiri* as species (Chen *et al.* 2008; Miguel *et al.* 2010). *Lactobacillus kefiri* strain was chosen due to *Lactobacillus kefiri* strains have significant proteolytic activity and protein hydrolysis ability (Anila *et al.* 2016).

In this study, antioxidant properties of casein, gluten, and soy protein hydrolysates are investigated after hydrolysing with an enzyme (because of the lack of solubility) and microbial (*Lactobacillus kefiri)* fermentation. Additionally, overexpressed proteins from microbial fermentation were identified.

MATERIAL AND METHODS

Materials. *Lactobacillus kefiri* (NRRL-B-1839) was provided from the United States Department of Agriculture Research, Education and Agricultural Research Service, ARS Culture Collection (NRRL). Gluten and soy protein were purchased from Sigma-Aldrich. Casein was obtained from skim milk. Digestive enzyme supplement product (DESP, contains DPPIV and protease was purchased from Enzymedica. The enzyme complex consists of DPPIV (1000DPU), amylase (15000 DU), protease (95000 HUT) and glucoamylase (15AGU).

Pre-culture and shake flask culture conditions. *Lactobacillus kefiri* was inoculated (as 5% v/v) into MRS broth, a pre-culture medium. Incubations were performed at 30°C for 48 hours. The inocula (10 ml) were cultured in 250 ml shake flasks (with 50 ml working volume) on an orbital shaker with 100 rpm at 30°C for 48 hours.

The production of casein. For a preparation was used 1000 ml of UHT milk. Milk was transferred into a beaker and heated up to 40 °C. When the temperature of milk reached 40 °C, acetic acid (10%, v/v) was added drop by drop and while stirring. Meanwhile, casein micelles were collected from the beaker. This process continued until the pH of milk reached 4.6. All the casein protein was dried at 50 °C and under vacuum.

Preparation of growth media with gluten, soy protein, and casein. The ratio of protein and glucose in growth media was calculated by considering C and N balance in MRS broth. Therefore, growth mediums were prepared with gluten (GMSG), soy protein (GMSS) and casein (GMSC). The GMSG contains 3 g of gluten and 0.2 g of glucose, GMSS contain 3 g of soy protein and 0.2 g of glucose, and GMSC contains 3 g of casein and 0.2 g of glucose.

Gluten, soy protein, and casein were treated with dissolved DESP in water (pH 6.5) at 37 °C for 2 h at 100 rpm on an orbital shaker for protein digestion and then sterilized and added by sterile glucose solution.

Preparation of strain and fermentation. *L. kefiri* strain was inoculated (as 5%, v/v) to the corresponding

medium with casein, soy protein, or gluten protein. The inoculum was cultured in 250 ml shake flasks (with 100 ml working volume) at 100 rpm at 30°C for 144 h, and samples were taken in every 48 h during the fermentation. The optical density of the strain was assessed by measuring absorbance at 660 nm by using spectrophotometer T80 UV/VIS (PG Instruments, UK). Each sample was collected by centrifugation at 8000 g for 15 minutes.

In vitro antioxidant activity of samples by 2,2, diphenyl 1-picryl hydrazyl (DPPH) radical scavenging assay. The 2,2, diphenyl 1-picryl hydrazyl (DPPH) radical scavenging and total reducing power (*RP*) assays have been widely used to determine antioxidant activity of various food samples, especially for the plant extracts. In the DPPH assay, samples (200 μl) were diluted with ddH_2O in order to prepare the different concentrations. After the addition of 0.2 mM of DPPH solution (600 μl), each sample was vortexed and incubated under dark at room temperature for 30 minutes. Aliquots of 200 μl of the samples were taken into 96-well-plate and the absorbance was measured at 570 nm against the blank (200 μl ddH_2O). The DPPH-scavenging activity was calculated by the following Equation (1):

$$DPPH\ (\%) = [1 - (A_s/A_b)] \times 1000 \tag{1}$$

where: A_s – absorbance of sample; A_b – absorbance of blank

Half-inhibition concentration (IC_{50}) showed the concentration of the sample at which 50% of absorbance at 570 nm was suppressed relative to the blank (Aytekin *et al*. 2011). In the DPPH assay, an antioxidant scavenges the free radicals by hydrogen donation.

Total reducing power (RP) assay. Different concentrations of samples were prepared in (1 ml) and mixed with 0.2 M sodium phosphate buffer (1 ml, pH 6.6). The reaction was initiated by addition of potassium ferricyanide (1%, 1 ml). After 20-min incubation at 50°C, 1 ml of trichloroacetic acid (10%) was added to stop the reaction, and the mixture was centrifuged. Supernatant was diluted with deionized water (1:1, v/v) and 0.2 ml of ferric chloride (0.1%) was added. After a 5-min incubation, absorbance was measured at 700 nm against the blank. A higher absorbance indicates a higher reducing power (Rice-Evans *et al*. 1997). In the RP assay, antioxidant compounds convert the oxidized form of iron (Fe^{+3}) in ferric chloride to ferrous (Fe^{+2}).

Degree of hydrolysis (DH). *o*-phthaldialdehyde (OPA) and serine were used as reagent and standard

for the *DH* analyses, respectively. Spectrophotometer readings were performed at 340 nm using deionized water as the control (Nielsen *et al*. 2001). The *DH* was calculated using the Equations (2, 3 and 4):

$$Serine\text{-}NH_2 = [(OD_{sample} - OD_{blank}) / (OD_{standard} - OD_{blank})] \times 0.9516 \times 0.1 \times (100/C) \tag{2}$$

$$h = [(serine\text{-}NH_2 - \beta)/\alpha]/g \tag{3}$$

$$DH\ (\%) = h/h_{tot} \times 100 \tag{4}$$

where: serine-NH_2 – standard protein in this analysis, milliequivalent value for Serin-NH_2 is 0.9516; *C* – total protein amount (g) in the sample; *h* – cleaved peptide bonds; α and β – constants for different proteins; h_{tot} – total number of peptide bonds protein equivalent dependent on amino acid composition of the sample

The h_{tot}, α, and β values were 7.8, 0.970, and 0.342 for soy, 8.3, 1.0, and 0.4 for gluten, and 8.2, 1.039, and 0.383 for casein, respectively.

Determination of protein amount. The protein concentration was calculated with the bicinchoninic acid assay (BCA), which was performed according to the manufacturer's protocol, Protein Assay Reagent Kit (Pierce; Thermo Scientific, USA).

Protease activity. Glycine-NaOH buffer (0.2 M) was prepared and mixed with 100 μl of 0.65% casein solution and 100 μl CSM, GSM, and SSM samples from microbial fermentation. After a 30-min incubation at 40°C, 0.11 M TCA reagent was added to each tube to stop the reaction. The mixture was centrifuged at 6700 g (10 000 rpm) for 10 minutes. Sodium carbonate (500 μl) and folin-ciocalteu phenol reagent (200 μl) were added to the supernatant (500 μl). Absorbance was measured at 660 nm. A standard curve was measured by *L*-tyrosine. Protease activity was calculated by the following Equation 5.

$$Enzyme\ (U/ml) = (Tyr \times V_t)/(t \times V_1 \times V_2) \tag{5}$$

where: Tyr – tyrosine equivalents release (μmol); V_t – total volume (ml); *t* – time of assay as per the unit definition (min); V_1 – volume of enzyme (ml); V_2 – volume used in colorimetric determination (ml)

SDS-PAGE analysis. The samples from microbial fermentation were directly examined with sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie brilliant blue R250 staining by following Laemmli (1970). Mo-

lecular weight marker proteins (molecular weight standard 5–250 kDa, Pierce) were used as references.

Mass spectrometry. Gel pieces containing related protein bands (the bold black single bands at 144 h for every gel represented as MS-1, MS-2 and MS-3) were excised from SDS-polyacrylamide gel and rinsed with a washing solution (methanol and acetic acid, 10:1, v/v). After destaining by ammonium carbonate (50 mM) and acetonitrile, the gel pieces were dried in 200 µl acetonitrile and then reduction and alkylation of proteins were completed with DTT and iodoacetamide, respectively. Trypsin solution was applied to the gel pieces for protein digestion at 37°C for 16 hours. Peptide extraction from gel pieces was performed with 50% acetonitrile and 5% formic acid.

The samples were analysed for protein identification by using Absciex MALDI-TOF/TOF 5800 system (AB Sciex, USA). Peak data were analysed with Mascot using streamline software, Protein Pilot (Absciex, USA). Only significant hits as defined by the Mascot probability analysis ($P < 0.05$) were accepted.

RESULTS AND DISCUSSION

Casein, gluten, and soy protein were treated with DESP to be hydrolysed and inoculated with *Lactobacillus kefiri*. DESP are generally used to enhance digestion of foods containing gluten and casein, and it is also providing enzyme support for gluten free/casein free diets. Growth constants of *Lactobacillus kefiri* in casein, gluten and soy protein media were measured and the maximum specific growth rate of *L. kefiri* strains was found as 0.036 h^{-1} in casein supplemented media (CSM). Casein plays a critical role for *L. kefiri* to turn milk into the kefir. *L. kefiri* breaks down the casein with the activity of cell wall-bound proteinase and harnesses the products for its own growth (ELFAHRI *et al*. 2014). Comparing with other resources, gluten (0.015 h^{-1}) and soy protein (0.029 h^{-1}), could not be utilized as efficient as casein by the strain.

The results of the antioxidant activity demonstrated that DPPH radical scavenging activity of casein increased from 1.58 ± 0.041% to 9.52 ± 0.057% after DESP treatment, and gradually rose up to 29.33 ± 0.015% through microbial fermentation (Figure 1). KUMAR *et al*. (2016) studied the effect of the hydrolysis time on camel milk casein and its antioxidant properties. The researchers found that DPPH and *RP* values of the casein were significantly increased by enzymatic hydrolysis. Because of a similar increasing pattern
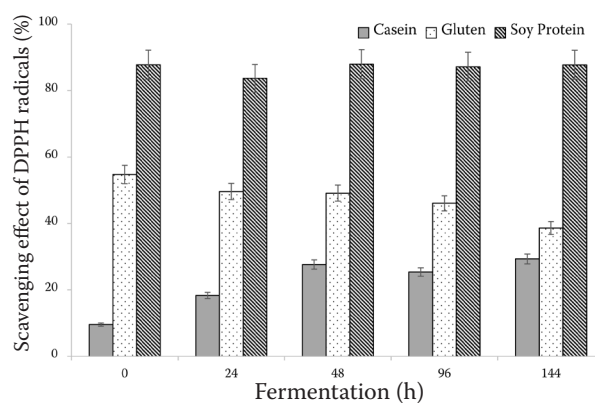

Figure 1. DPPH activity of casein, gluten and soy protein hydrolysates

with DPPH and the density of *Lactobacillus kefiri* strain, it is thought that after the DESP treatment, remained protein fragments might be hydrolysed to the antioxidative peptides by proteases from the strains. SAVIJOKI *et al*. (2006) reported that LAB is needed an exogenous source of amino acids or peptides, which are provided by the proteolysis of casein, the main protein and source of amino acids in milk. Therefore, LAB expresses various membrane-bound proteinases or intracellular peptidases that degrade the protein into shorter peptides and amino acids. However, extracellular protease activities of CSM, GSM, and SSM were not changed during the microbial fermentation and remained at 0.424 U/ml, 0.490 U/ml, and 0.496 U/ml, respectively.

Reducing the power of casein increased 1.6 times by DESP treatment (data not shown), and it showed a tendency to fall at the end of the microbial fermentation (Figure 2). The obtained results indicate that casein contains peptides which can donate hydrogen and electrons two times less than soy protein. MORITA *et al*. (1997) and YAMAZAKI (1982) studied amino
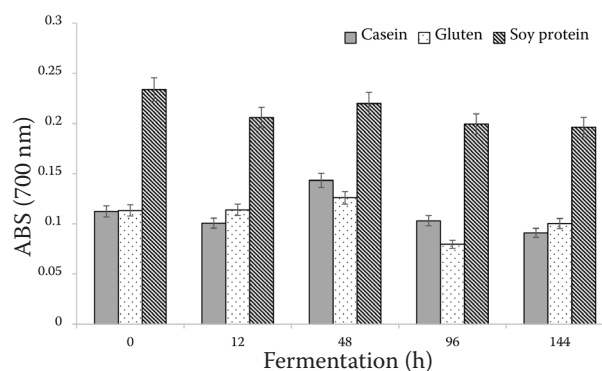

Figure 2. Reducing power of casein, gluten and soy protein hydrolysates

acid compositions of casein, soybean, potato, and rice, and found that soybean comprising the highest amount of glutamic acid (Glu), cysteine (Cys), and glycine (Gly), which have high ferric reducing antioxidant power values.

After DESP treatment, DPPH radical scavenging activity of gluten increased up to 54.72% and then slightly decreased during the fermentation period in parallel to the cell density. There are minor fluctuations *RP* values of gluten during the fermentation period.

Among three protein samples in the study (Table 1), soy protein exhibited the highest DPPH radical scavenging activity (58.11%) and DESP treatment elevated this value to 87.74%. Udenigwe and Aluko (2011) reported greater DPPH activities for histidine (His) and aspartic acid (Asp), of which amounts are enriched in soy protein compared to gluten and casein (Morita *et al.* 1997). The reducing power assay (0.234) was also revealed to have minor fluctuations during the fermentation. Oliveira *et al.* (2014) reported DPPH and *RP* values of soy protein hydrolysates as 78.06 and 0.213%, respectively. These findings correlate with our results for the present study. The peptides of soy protein suggest an application on the reduction of oxidized intermediates of lipid peroxidation in foods. According to the results of DPPH scavenging activity of soy protein, it has not been affected by the microbial fermentation. DPPH scavenging activity depends on the amino acid contents of proteins. Soy protein has the highest capacity than the other proteins, and the cell density of the strain is similar to the casein (Figure 1).

DPPH IC$_{50}$ values of the gluten and soy protein. DPPH radical scavenging activity values of gluten and soy protein samples were measured during the fermentation period, and then IC$_{50}$ (the concentration of the sample required to inhibit 50% of radical) values were calculated. However, casein was not included in this study since its IC$_{50}$ values were under the 50%. Soy protein displayed higher antioxidant activity (2.64 mg/ml) than gluten (15.71 mg/ml) at the initial stage of the microbial fermentation. Zhang *et al.* (2018a) reported that IC$_{50}$ value of digested

soybean hydrolysate was measured as 4.22 mg/ml. In another study of the same researchers showed that IC$_{50}$ value of soybean hydrolysate below from 3 kDa was found as 2.56 mg/ml (Zhang *et al.* 2018b). The present study has been supported by these data. In the later stages of fermentation, antioxidant activities of the proteins changed according to the growth phase. *L. kefiri* showed a significant growth while the antioxidant activities of protein resources, gluten and soy protein, were not changed during the first 48 hours. The antioxidant activity gradually increased after the exponential phase in gluten, which might be affected by cell death. Similarly, IC$_{50}$ values of soy protein slightly rose up to 96 h, and then remained stable (data not shown).

Determination of degree of hydrolysis. OPA method was performed to digested GMSG, GMSS, GMSC mixtures after enzymatic digestion. The protein hydrolysis of samples was observed at high values. The most hydrolysed protein sample was monitored as GMSS that was reached 91.4% hydrolysation. While, hydrolysis degrees of GMSG and GMSC were 81.2 and 87.8%, respectively. The results indicated that all digested mixtures were successfully hydrolysed by the process mentioned above. The data were supported by SDS-PAGE analysis (Figure 3). Kong *et al.* (2007) reported that the degree of hydrolysis value from digested wheat gluten reached 63.7%. Hanafi *et al.* (2018) studied with Alcalase digested green soybean hydrolysate and found the degree of hydrolysis value 61.6%. The difference between data might be related to the preferred enzyme types, enzyme-substrate ratio and the condition of the pretreated substrate.

Determination of the molecular weight of proteins and mass spectrometry analyses. The protein samples from GMSG, GMSS, GMSC, and corresponding enzymatic hydrolysates of these proteins and microbial fermentation were separated on an SDS-polyacrylamide gel followed by Coomassie brilliant blue R250 staining to determine protein distribution profiles. After the enzymatic hydrolysis (0th), the high molecular weight-protein band was not detected for casein, gluten, and soy protein. Gel electrophoresis

Table 1. The amount of protein in supplemented media (mg/ml)

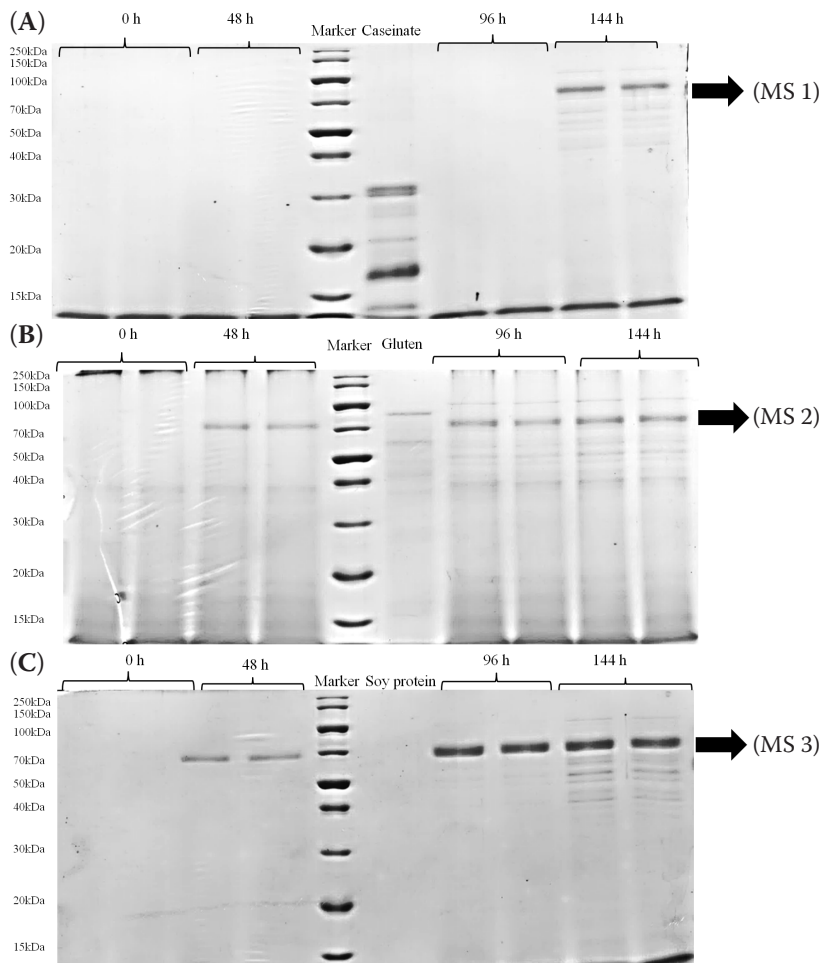| Medium	DESP treatement		Fermentation time (h)				
	before	after	24	48	96	144	192
Casein	–	13.63 ± 0.40	13.84 ± 0.22	10.87 ± 0.73	11 ± 0.19	8.94 ± 0.75	10.09 ± 0.16
Gluten	–	17.85 ± 0.52	14.7 ± 0.50	14 ± 0.35	15.11 ± 0.58	16.43 ± 0.25	14.7 ± 0.58
Soy protein	–	11.62 ± 0.26	11.34 ± 0.34	10.87 ± 0.13	9.93 ± 0.09	10.12 ± 0.24	10.24 ± 0.19

Figure 3. Electrophoretic profiles of enzymatic hydrolysis and microbial fermentation of casein (**A**), gluten (**B**) and soy protein hydrolysates (**C**)

results showed that all the proteins (casein, gluten, and soy protein) were totally digested after DESP treatment (Figure 3). Therefore, any protein bands were detected at 0th of fermentation in Figure 3. A couple of new protein bands were detected for gluten and soy protein samples after 48 h of microbial fermentation. Our results revealed an increase in the amount of the protein throughout the microbial fermentation. Particularly, the new protein appeared at 144 h in GMSC aroused our interest. In order to identify these new proteins, mass spectrometry-based proteomic analysis employing in-gel tryptic digestion was performed. According to these analyses, peptides belonging to elongation factor G (EF-G accession no: EFG_NEOSM), chaperone protein DNA-K (accession no: DNAK_ARCB4), and DNA-J (accession no: DNAJ_ACTP2) were found to be significant with mascot scores above 40 (Table 2 and Figure 4). EF-G, translocase functions as elongation factor during protein synthesis, where it is involved in the translocation of tRNA and mRNA down the ribosome (Shoji *et al.* 2009). In addition, it promotes the recycling of ribosome subunits in a GTP-dependent manner (Zavialov *et al.* 2005). Overall, the elevated expression of EF-G in our samples upon treatments might indicate an increased protein synthesis activity for the enzymes required to hydrolyse the proteins. On the other hand, DNA-K and DNA-J are members of molecular chaperone family proteins protecting newly synthesized proteins from aggregation, particularly during cellular stress conditions (Mayer 2010). Samples exposed to different treatments in our studies might induce this kind of responses so the *L. kefiri* adapts the applied conditions by changing the overall proteome.

These proteins along with the whole proteome of *L. kefiri* are needed to be characterized in detailed proteomic approaches from the samples enriched with advanced purification and separation techniques, including molecular weight filtration and fractionation with ultra-high-performance liquid chromatography coupled with high-resolution tandem mass spectrometry. The outcome of these further characterization applications might unveil the strain-specific characteristics of corresponding hydrolytic enzymes and peptides.

Table 2. List of proteins and peptides identified by MALDI-TOF/TOF MS after electrophoretic separation

Sample	Accession	Description	OS	GN	PE	SV	Mass (m/z)	Mascot score	Peptides + modification	Observed	Start	End
MS-1	EFG_NEOSM	Elongation factor G	*Neorickettsia sennetsu* (strain Miyayama)	fusA	3	1	76 088	40	M.SASSVELEK.I	949, 4991	2	10
									-.MSASSVELEKIR.N + Oxidation (M)	1365, 6876	1	12
									R.MLLMHANSREDVK.S	1543, 7042	355	367
									R.IGRMLLMHANSREDVK.S + 2 Oxidation (M)	1901, 9084	352	367
									K.STAEQEKMALAVARLCAEDPSLK.V + Carbamidomethyl (C)	2518, 2712	416	438
MS-2	DNAK_ ARCB4	Chaperone protein dnaK	*Arcobacter butzleri* (strain RM4018)	dnaK	2	1	67 456	52	K.VGYKIVDR.N	949, 5117	89	96
									K.SLTRAKFESMTEK.L + Oxidation (M)	1543, 7377	293	305
									R.IINEPTAASLAYGLDK.K	1675, 8247	167	182
									K.VIGIDLGTTNSCVAVYENGEAK.I + Carbamidomethyl (C)	2310, 0923	4	25
MS-3	DNAJ_ACTP2	Chaperone protein dnaJ	*Actinobacillus pleuropneumoniae* serotype 5b (strain L20)	dnaJ	3	1	40 791	46	K.GASENDIKRAYK.R	1351, 6434	15	26
									K.IEKPCKSCHGDGR.V + 2 Carbamidomethyl (C)	1543, 7113	198	210
									R.RQQGFFVTEAVCPSCHGSGKK.I	2266, 1267	177	197
									R.GDDLRYDIEISLEEAVKGCK.K + Carbamidomethyl (C)	2310, 0459	119	138
									R.GGYAGDLICKVVVETPVALNDEQK.D	2518,2859	320	343

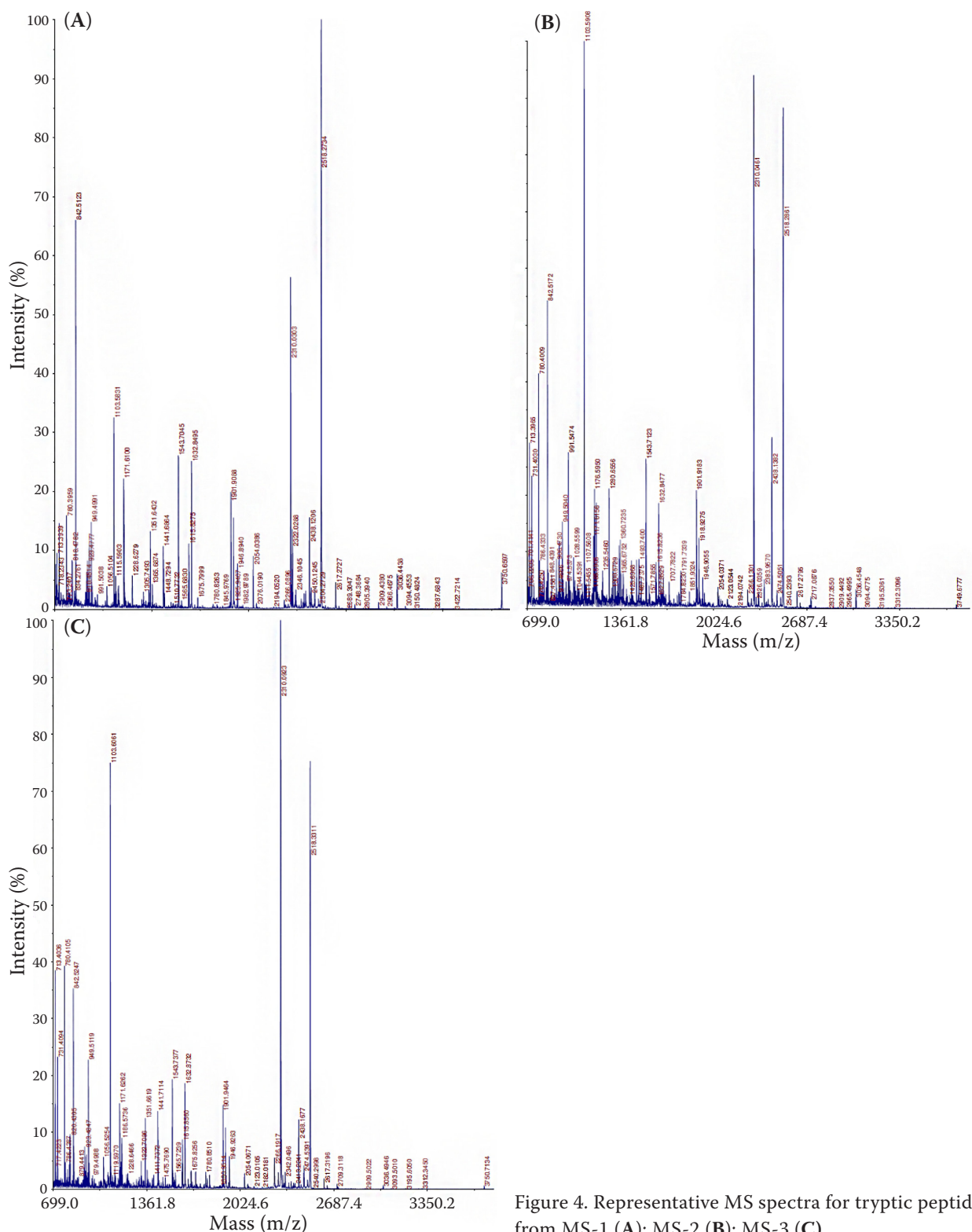OS – microorganism; GN – gene name; PE – protein existence; SV – sequence version

Figure 4. Representative MS spectra for tryptic peptides from MS-1 (**A**); MS-2 (**B**); MS-3 (**C**)

CONCLUSIONS

Casein, gluten and soy protein has a great potential to be used in the food industry. These proteins can be hydrolysed through enzymatic or microbial processes to produce peptides displaying antioxidant activities. According to our results, we unrevealed a strong correlation between antioxidant activities and enzymatic hydrolysis of casein, gluten, and soy protein. The results indicated that hydrolysis of casein was sig-

nificantly enhanced by microbial fermentation along with elevated DPPH radical scavenging activities. Further studies are needed to characterize the enzyme expression profile from different *L. kefiri* strains.

References

Anila K., Kunzes A., Bhalla T.C. (2016): *In vitro* cholesterol assimilation and functional enzymatic activities of putative probiotic *Lactobacillus* sp*.* isolated from fermented foods/beverages of North West India. Journal of Nutrition and Food Sciences, 6: 1–5.

Aytekin A.O., Morimura S., Kida K. (2011): Synthesis of chitosan–caffeic acid derivatives and evaluation of their antioxidant activities. Journal of Bioscience and Bioengineering, 111(2): 212–216.

Chen H.C., Wang S.Y., Chen M.J. (2008): Microbiological study of lactic acid bacteria in kefir grains by culture-dependent and culture-independent methods. Food Microbiology, 25(3): 492–501.

Cho D.Y., Jo K., Cho S.Y., Kim J.M., Lim K., Suh H.J., Oh S. (2014): Antioxidant effect and functional properties of hydrolysates derived from egg-white protein. Korean Journal for Food Science of Animal Resources, 34: 362.

Elfahri K.R., Donkor O.N., Vasiljevic T. (2014): Potential of novel *Lactobacillus helveticus* strains and their cell wall bound proteases to release physiologically active peptides from milk proteins. International Dairy Journal, 38: 37–46.

Gaspar A.L.C., de Góes-Favoni S.P. (2015): Action of microbial transglutaminase (MTGase) in the modification of food proteins: A review. Food chemistry, 171: 315–322.

Hanafi M.A., Hashim S.N., Chay S.Y., Ebrahimpour A., Zarei M., Muhammad K., Abdulhamid A., Saari N. (2018): High angiotensin-I converting enzyme (ACE) inhibitory activity of alcalase-digested green soybean (*Glycine max*) hydrolysates. Food Research International, 106: 589–597.

Kong X., Zhou H., Qian H. (2007): Enzymatic hydrolysis of wheat gluten by proteases and properties of the resulting hydrolysates. Food Chemistry, 102: 759–763.

Kumar D., Chatli M.K., Singh R., Mehta N., Kumar P. (2016): Enzymatic hydrolysis of camel milk casein and its antioxidant properties. Dairy Science and Technology, 96(3): 391–404.

Laemmli U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680.

Li-Chan E.C. (2015): Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients. Current Opinion in Food Science, 1: 28–37.

Mayer M.P. (2010): Gymnastics of molecular chaperones. Molecular cell, 39: 321–331.

Matoba T. (2002): How does the radical-scavenging activity of soy protein food change during heating. Daizu Tanpakushitsu Kenkyu, 5: 47–50.

McMahon D.J., Oommen B.S. (2013): Casein micelle structure, functions, and interactions. In: McSweeney P.L.H., Fox P.F. (eds): Advanced Dairy Chemistry. Boston, Springer International Publishing: 185–209.

Miguel M.G.D.C.P., Cardoso P.G., de Assis Lago L., Schwan R.F. (2010): Diversity of bacteria present in milk kefir grains using culture-dependent and culture-independent methods. Food Research International, 43: 1523–1528.

Morita T., Oh-hashi A., Takei K., Ikai M., Kasaoka S., Kiriyama S. (1997): Cholesterol-lowering effects of soybean, potato and rice proteins depend on their low methionine contents in rats fed a cholesterol-free purified diet. The Journal of Nutrition, 127: 470–477.

Nielsen P.M., Petersen D., Dambmann C. (2001): Improved method for determining food protein degree of hydrolysis. Journal of Food Science, 66: 642–646.

Nishinari K., Fang Y., Guo S., Phillips G.O. (2014): Soy proteins: A review on composition, aggregation and emulsification. Food Hydrocolloids, 39: 301–318.

Oliveira C.F., Coletto D., Correa A.P.F., Daroit D.J., Toniolo R., Cladera-Olivera F., Brandelli A. (2014): Antioxidant activity and inhibition of meat lipid oxidation by soy protein hydrolysates obtained with a microbial protease. International Food Research Journal, 21: 775–781.

Payne P.I. (1987): Genetics of wheat storage proteins and the effect of allelic variation on bread-making quality. Annual Review of Plant Physiology, 38: 141–153.

Pihlanto A. (2006): Antioxidative peptides derived from milk proteins. International Dairy Journal, 16(11): 1306–1314.

Rajapakse N., Mendis E., Jung W.K., Je J.Y., Kim S.K. (2005): Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. Food Research International, 38: 175–182.

Rice-Evans C., Miller N., Paganga G. (1997): Antioxidant properties of phenolic compounds. Trends in Plant Science, 2: 152–159.

Savijoki K., Ingmer H., Varmanen P. (2006): Proteolytic systems of lactic acid bacteria. Applied Microbiology and Biotechnology, 71: 394–406.

Shewry P.R., Halford N.G., Tatham A.S. (1992): High molecular weight subunits of wheat glutenin. Journal of Cereal Science, 15: 105–120.

Shoji S., Walker S.E., Fredrick K. (2009): Ribosomal translocation: one step closer to the molecular mechanism. ACS Chemical Biology, 4: 93–107.

Udenigwe C.C., Aluko R.E. (2011): Chemometric analysis of the amino acid requirements of antioxidant food protein hydrolysates. International Journal of Molecular Sciences, 12(5): 3148–3161.

Utsumi S., Matsumura Y.A.S.U.K.I., Mori T. (1997): Structure-function relationships of soy proteins. In: Damodaran S., Paraf A. (eds): Food Proteins and Their Applications. New York-Marcel Dekker: 257–292.

Üstün Ö., Öngen G. (2012): Production and separation of dipeptidyl peptidase IV from *Lactococcus lactis*: scale up for industrial production. Bioprocess and Biosystems Engineering, 35: 1417–1427.

Villegas J.M., Picariello G., Mamone G., Turbay M.B.E., de Giori G.S., Hebert E.M. (2014): Milk-derived angiotensin-I-converting enzymeinhibitory peptides generated by *Lactobacillus delbrueckii* subsp. *lactis* CRL 581. Peptidomics, 1.

Wang J., Zhao M., Yang X., Jiang Y. (2006): Improvement on functional properties of wheat gluten by enzymatic hydrolysis and ultrafiltration. Journal of Cereal Science, 44: 93–100.

Yamazaki K.I., Takao S., Yamamoto K.: Process for preparing modified protein compositions. Japan. Patent EP 0047879 A1, 1982.

Zavialov A.V., Hauryliuk V.V., Ehrenberg M. (2005): Splitting of the posttermination ribosome into subunits by the concerted action of RRF and EF-G. Molecular cell, 18: 675–686.

Zhang Q., Tong X., Qi B., Wang Z., Li Y., Sui X., Jiang L. (2018a): Changes in antioxidant activity of Alcalase-hydrolyzed soybean hydrolysate under simulated gastrointestinal digestion and transepithelial transport. Journal of Functional Foods, 42: 298–305.

Zhang Q., Tong X., Sui X., Wang Z., Qi B., Li Y., Jiang, L. (2018b): Antioxidant activity and protective effects of Alcalase-hydrolyzed soybean hydrolysate in human intestinal epithelial Caco-2 cells. Food Research International, 111: 256–264.