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# Fungal metalloprotease generate whey-derived peptides that may be involved in apoptosis in B16F10 melanoma cells

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**Abstract**

Proteases are enzymes that act in the hydrolysis of proteins and have several industrial applications. Moreover, proteases have gained prominence as enzymes for the generation of bioactive peptides from the hydrolysis of different protein sources. Milk is the most studied protein source to obtain peptides due to its nutritional and physiological effects and has been studied as complementary therapeutic approaches for the cancer treatment, interacting specifically with cancer cells, consequently fewer side effects. The ability of *Eupenicillium javanicum* metalloprotease to generate whey-derived peptides with antioxidant activity has already been demonstrated. For this reason, we thus hypothesized that whey-derived peptides from *Eupenicillium javanicum* metalloprotease hydrolysis could also have a potential against melanoma cell lines. In this study, B16F10 melanoma cells were treated for 72 h with whey-derived peptides and the effects on cell viability were determined. Moreover, the protein profiles of the treated and nontreated cells were compared in proteomic assay and mass spectrometry analyzes. Whey-derived peptides impaired about 62% cell viability, and proteomic approach associated this behavior to modulate proteins involved in proliferation, energy, apoptosis, metastatic and malignancy rates. This study describes the relevance of microbial enzymes in generation of whey-derived peptides with biological activity against melanoma cells.

**Keywords:** bioactive peptide; *Eupenicillium javanicum*; microbial protease; proteomic.

**Practical Application:** The treatment with whey-derived peptide showed a preliminary potential as alternative/complementary therapy in the fight against cancer, but more elucidative studies should be carried out to define precisely how the effect of these molecules occurs in the target cell.

## 1 Introduction

The use of enzymes in industry has increased annually and proteases represent the largest group of commercially available enzymes worldwide due to their wide range of applications in food and beverage, cleaning products, animal feed, and others (Razzaq et al., 2019). Proteases are enzymes that catalyze the protein hydrolysis and constitute a complex group of enzymes that differ in properties such as substrate specificity, catalytic mechanism and stability profile (Manfredini et al., 2021). Microbial enzymes has been widely explored for their potential and filamentous fungi are responsible for the production of most enzymes used worldwide (Manfredini et al., 2021). However, the search for alternative sources to produce microbial enzymes (Worsztynowicz et al., 2020; Ahangari et al., 2021) can be useful in obtaining bioactive peptides (BAPs) capable of acting in the control of different diseases.

BAPs are 2-20 amino acid-long fragments of natural proteins, releasing bioactive molecules upon hydrolyzation (Udenigwe & Aluko, 2012). In this context, proteases have gained prominence in food hydrolysis, because it is a very useful tool for BAPs production, but its use requires a broad knowledge of peptide properties, such as hydrolysis degree, molecular weight and composition of amino acids (Martínez-Medina et al., 2019). BAPs have an activity related to the binding capacity of specific cell surface receptors, triggering intracellular effects, acting as hormones, neurotransmitters, growth factors, and ion channel ligands. This specificity confers fewer side effects and more efficacy than chemotherapy (Fosgerau & Hoffmann, 2015).

Milk-derived peptides have been studied as promise molecules for designing nutraceuticals, functional foods, or other pharmaceutical products (Mohanty et al., 2016). Whey is the supernatant of casein coagulation, and it has been previously considered as waste by the dairy industry (Patel, 2015) and they are mainly composed of beta-lactoglobulin and alpha-lactalbumin (Mohanty et al., 2016). Whey-derived bioactive peptides have been studied as alternatives for several disturbances, such as, antitumor activity (Yoo et al., 1997), antihypertensive agents (Michelke et al., 2017), inhibiting dipeptidyl peptidase-IV, an enzyme related to type 2 diabetes (Lacroix et al., 2016), antibacterial

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activity against *Escherichia coli*, *Bacillus cereus*, *Salmonella typhimurium*, and *Staphylococcus aureus* (Osman et al., 2016) and antioxidant activity with strong DPPH radical quenching effect (Rocha et al., 2017).

The filamentous fungus *Eupenicillium javanicum* is capable to produce a metalloprotease (Hamin Neto et al., 2017), can be an interesting alternative source to generate peptides with bioactivity (Hamin Neto et al., 2019). The aim of this study was to investigate whey proteins in detail for their potential as cheaper and safer alternative anticancer treatments, which also possess additional nutritive value and beneficial physiological properties. We hypothesized that whey-derived peptides, generated by a fungal protease from *E. javanicum* would exhibit a potential chemopreventive effect against B16F10 melanoma cell lines.

## 2 Materials and methods

### *2.1 Fungal protease, substrate and hydrolysis*

Metalloproteases were produced by the fungus *Eupenicillium javanicum* under a condition-optimized submerged bioprocess according to a previously described protocol (Hamin Neto et al., 2013), and purified by two separation processes (Hamin Neto et al., 2019). The bovine milk-derived whey substrate used in this study was kindly provided by a dairy product manufacturer (Serralat Laticínios LTDA, Serrana, Brazil). The whey was concentrated by the tangential filtration system FlexStand™ (GE Healthcare) with a molecular weight cutoff membrane of 10 kDa. The concentrated samples were frozen, lyophilized, and stored at 4 °C. The substrate solution was prepared using ultra-pure water at a 5% (w/v) concentration for protein hydrolysis. The whey solution was hydrolyzed in a proportion of 1.0 µg of enzyme per 20 µg of substrate; and the reaction was conducted at 45 °C and 200 rpm for 12 h. Following the reaction, proteins were denatured by heating (96 °C, 15 min) and centrifuged (10,000 × *g*, 10 min, 10 °C). Supernatants were frozen, lyophilized, and stored at -80 °C.

### *2.2 Protein fractionation by C18 reverse-phase chromatography, Mass Spectrometry and in silico analysis*

The nonhydrolyzed whey (control) and hydrolyzed samples were prepared at 80 $mg.mL^{-1}$, centrifuged (10,000 × *g*, 10 min, 25 °C) and 8 mg of samples, control or hydrolyzate, were subjected to C18 reverse phase chromatography (ACE 5 C18-300). The resin was equilibrated with trifluoroacetic acid 0.1% and the elution gradient was 0 - 80% acetonitrile, using a chromatograph AKTA purifier (GE Healthcare) and the Unicorn 5.20. software. The protein profiles of the samples were determined and fractions belonging only to the hydrolyzed profile were selected for mass spectrometry. The samples were prepared on a column tip containing reverse-phase resin (POROS R2; PerSeptiveBiosystems, Framingham, MA, USA) equilibrated with 0.2% formic acid, the peptides were eluted in 60% methanol solution in 5% formic acid, dried in a vacuum concentrator and prepared for LC-ESI-Q-TOF-MS. The peptides were analyzed using a Quadrupole-Time of Flight electrospray mass spectrometer (Q-TOF-Ultima; Waters, Manchester, UK) coupled to a nanoAccquity capillary chromatography system using reverse-phase 10 cm × 75 µm i.d. 3 µm particle technology (Waters, Manchester, UK). Mass spectra were collected using data-dependent acquisition (DDA) of the top three ions in both MS and MS/MS modes and processed using the MassLynx software v. 4.1 (Waters, Manchester, UK) to generate a peak list file that was submitted for database analysis using the MASCOT v.2.4.2 software and a specific database tolerance of 1.2 Da for precursor ions and 0.8 Da for product ions. No fixed modifications or variable modifications, such as methionine oxidation, were included. In addition, all enzymes were excluded and only a single missed cleavage was permitted.

The *in silico* analysis was performed to predict the physicochemical characteristics and bioactivity potential of the identified peptides. The sequences were analyzed using AntiCP (Tyagi et al., 2013) and iACP (Chen et al., 2016) for anticancer potential and also in Milk Bioactive Peptide Database (MBPD) (Nielsen et al., 2017).

### *2.3 Melanoma cell (B16F10) culture and treatment with WDP*

The B16F10 murine melanoma cell line (ATCC: CLR-6475) was used as a melanoma model. Cells were cultured in a 75-$mm^2$ bottle in Ham's F-10 medium (Gibco; pH 6.9) supplemented with 1.2 $g.L^{-1}$ sodium bicarbonate, 10% fetal bovine serum (FBS; Gibco), and 1% streptomycin/penicillin (w/v) (Sigma Aldrich®) in a humid atmosphere at 5% $CO_2$ and 37 °C. When B16F10 murine melanoma reached the 70% confluency, the cells were washed with phosphate-buffered saline (PBS), trypsinized, resuspended and then distributed into 96-well plates at 3,000 cells per well. The culture medium was then changed, and the B16F10 cells were cultured without FBS supplementation for 16 h. A whey-derived peptide solution was added to the cell cultures at 4 $mg.mL^{-1}$ and incubated for 48 h. Cells treated with the peptide solvent (water) were included as a negative control, and 5% DMSO was included as a positive control. After incubation, cell viability was assessed based on resazurin salt reduction, as previously described (O'Brien et al., 2000). The absorbance was measured on a Synergy 2 Multi-Mode Microplate Reader (Biotek), with an excitation at 530/25 nm and an emission of 590/35 nm. For the cell viability assay, all data were expressed as means ± standard deviations and were statistically analyzed by two-way ANOVA followed by Bonferroni posttests, and $p < 0.05$ was considered significant.

### *2.4 Melanoma cell (B16F10) proteomic assay: tryptic digestion and sample purification*

B16F10 cells ($1 \times 10^6$ cells) cultured on a 100-mm plate cells were treated with 4 $mg.mL^{-1}$ of WDP or water (negative control) for 48 h. Then, the cells were washed with PBS, trypsinized, and centrifuged twice (1,200 × *g*, 5 min, 20 °C). Cells were lysed with a protease inhibitor cocktail (cOmplete Ultra, Roche) in 0.5 M Tris-HCl (pH 7.5), 0.1 M KCl, 0.05 M EDTA (pH 8.0), 0.7 M sucrose and 15 µL of beta-mercaptoethanol and subsequently in according to Hurkman & Tanaka (1986). The precipitate was resuspended in buffer (7.0 M urea, 2.0 M thiourea, 10 mM dithiothreitol, and 0.01% Triton X-100) and protein concentrations were determined (Bradford, 1976). Fifty-micrograms of proteins from WDP-treated or non-treated

cells were denatured by heating at 80 °C for 15 min. Denatured samples were centrifuged (10,000 × *g*, 25 °C, 30 s) and 2.5 µL of 100 mM dithiothreitol (GE Healthcare, Amersham Place, UK) was added. The samples were heated at 60 °C for 30 min, cooled to room temperature and centrifuged (10,000 × *g*, 25 °C, 30 s). Then, 2.5 µL of 300 mM iodoacetamide (GE Healthcare, Amersham Place, UK) was added and the mixture was incubated in the dark at 30 °C for 30 min. For digestion, 10 µL of trypsin solution (Promega, Wisconsin, EUA) in 50 mM ammonium bicarbonate was added at an enzyme/protein ratio of 1:100, and the samples were incubated at 37 °C for 16 h. The reaction was stopped with 10 µL of 5% trifluoroacetic acid (Merck, Hohenbrunn, Germany). Samples were incubated at 37 °C for 90 min, centrifuged (14,000 × *g*, 6 °C, 30 min) and the supernatants were vacuum-dried at 30 °C. The dried samples were solubilized in 10 µL of 0.1% trifluoroacetic acid (TFA) and purified using reverse-phase Zip-Tip C18 column (Millipore), in according to manufacturer instructions, and eluted with 10 µL of 50% (v/v) Acetonitrile:$H_2O$ with 0.1% (v/v) TFA. The samples were concentrated by vacuum centrifugation at 30 °C.

### *2.5 Liquid chromatography-mass spectrometry*

The samples were resuspended in 32 µL of 20 mM ammonium formate (pH 10.0) and 8 µL of yeast alcohol dehydrogenase (SwissProt P00330, internal standard, 100 fmol. $\mu L^{-1}$). The peptides were sequenced on a Synapt G2 HDMS mass spectrometer (Waters, Manchester, UK) coupled to an Acquity UPLC MClass system with 2D technology (Waters). The peptides were captured on a Trap 2D Symmetry C18 column (5 µm, 180 µm × 20 mm) (Waters), separated on a first-dimension column, UPLC M-Class peptide BEH C18 (5 µm, 300 µm × 50 mm) (Waters) and an analytical column, Acquity UPLC M-Class peptide CSH C18 (1.7 µm, 75 µm × 150 mm) (Waters). The experiments were carried out in the HDMSE (independent data analysis) mode, which produces precursor ions and products in sequence. The scan time was 0.8 s in each mode, in the m/z range of 50 - 2,000.

### *2.6 Sequence determination and functional analysis of the identified proteins*

The mass spectral data were processed using ProteinLynx GlobalServer (PLGS) software version 3.1, with the reverse database of reference proteome *Mus musculus* (Proteome ID UP000000589) (The Uniprot Consortium, 2019). The processing parameters included automatic tolerance for precursors and product ions, a minimum of three corresponding fragment ions per peptide, a minimum of seven corresponding fragment ions per protein, and a minimum of two corresponding peptides per protein. A possible cleavage error by trypsin, carbamidomethylation of cysteine (fixed modification), oxidation of methionine (variable modification) and maximum false-positive discovery rate at 4%. All analyses were conducted in triplicate, and proteins were considered only if present in two or more replicates for the treatment and non-treatment groups. The concentration of each protein was normalized to the total protein in each replicate. For the mass spectrometry assay, statistical analysis was conducted using MetaboAnalyst 4.0. Student's t-test was used, and $p < 0.05$ was considered significant.

## 3 Results and discussion

### *3.1 Protein profile and WDP fractionation, mass spectrometry and in silico analysis*

In order to perform whey-derived peptide sample characterization and observe the difference between the protein profiles of intact and hydrolyzed whey, the samples were evaluated on C18 reverse phase chromatography. Four fractions were selected for identification by mass spectrometry (LC-MS) and 17 peptides were identified (Table 1).

In fraction 1, the proteins α-s1-casein and β-casein originated the identified peptides. The peptides from fractions 2 and 4 were derived from κ-casein and β-lactoglobulin, respectively. The peptides were analyzed in Anticp, iACP and MBPD. The peptides were analyzed by Anticp software in order to predict their physicochemical characteristics (Table 1). In MBPD were detected that 7 peptides have already been identified for having biological activity in MBPD: antioxidant, antimicrobial, ACE-inhibitory and immunomodulatory (Table 1). All peptides were also submitted to iACP analysis (Chen et al., 2016) and among the identified peptides, two of them could present anticancer potential, IIAEKTKIPAVF and KKIIAEKTKIPAVF, 95.8% and 97.9%, respectively (Table 1).

According to the iACP analysis, anticancer peptides showed a length of 12 to 14 amino acids in their composition and cationic nature. Amino acids such as Phe, Ile and Lys, were more abundant in anticancer peptides than non-anticancer peptides. Studies suggest that peptide selectivity is related to the anionic features of the cancer cell membranes such as greater cell surface and membrane fluidity compared to normal cells. These characteristics allow an improvement in lytic activity and binding capacity of anticancer peptides (Tyagi et al., 2013). Moreover, the sequence of amino acids could influence, because the interaction between peptide and cancer cell membrane could be due conformation (Chen et al., 2016). WDP sample showed peptides with several potential activities (Table 1), such as: antioxidant, antimicrobial, ACE-inhibitory, immunomodulatory and anticancer potential. The dual potential (anticancer and antimicrobial) of some peptides is known, as these peptides share similar properties, such as amphipathicity, hydrophobicity, and overall positive charge (Felício et al., 2017).

### *3.2 Melanoma cell (B16F10) viability assay*

B16F10 viability was evaluated after 24, 48, and 72 h compared to water treatment (negative control), DMSO (positive control) or WDP (pool of peptides). The WDP treatment after 24, 48 and 72 h has reduced the B16F10 cells viability in levels of 29.7 ± 3.8%, 37.7 ± 4.0% and 61.7 ± 5.1%, respectively (Figure 1).

According to Castro et al. (2009) hydrolyzates from bovine whey protein, inhibited the proliferation of B16F10 melanoma cell line and they related this result to higher caspase-3 expression, which induces apoptosis. Azevedo et al. (2012) synthesized a peptide INKKI and showed dose-response cytotoxicity selective for the B16F10 cell line, with caspase-3 mediated apoptosis induction and cell arrest in the G2/M phase. The authors hypothesized that the cationic properties of INKK might increase its binding to the

**Table 1**. Mass spectrometry (LC-MS) identification of peptides fractionated from C18 reverse phase chromatography of whey-derived peptides and Anticp, iACP and MBPD analyzes

| Frac. | Identified sequence/ bovine protein source* | pI* | Molecular mass* | MBPD Function threshold (%)** | Anticancer potential*** |
|---|---|---|---|---|---|
| 1 | GLPQEVLNENLLRFF/α-s1 casein | 4.54 | 1936.49 | Antioxidant (80%) | No |
| 1 | DVPSERYLGYLEQLLRLK/ α-s1 casein | 6.52 | 2321.01 | Antimicrobial (70%) | No |
| 1 | PSERYLGYLEQLLRLK/α-s1 casein | 8.83 | 2106.76 | | No |
| 1 | DVPSERYLGYLEQLL/α-s1 casein | 4.14 | 1908.43 | | No |
| 1 | DVENLHLPLPLLQSW/β-casein | 4.36 | 1960.52 | ACE-inhibitory (70%) | No |
| 1 | LLYQEPVLGPVRGPFPIIV/ β-casein | 6.35 | 2207.02 | ACE-inhibitory (100%) Immunomodulatory (90%) | No |
| 1 | TPVVVPPFLQPEVMGVS/ β-casein | 4 | 1883.5 | Antioxidant (70%) | No |
| 2 | TIASGEPTSTPTTEAVE/κ-casein | 3.68 | 1820.14 | | No |
| 2 | RHPHPHLSFMAIPPK/κ-casein | 11 | 1893.52 | | No |
| 2 | VATLEDSPEVIE/ κ-casein | 3.51 | 1430.71 | | No |
| 2 | FFSDKIAKYIPIQYVLSR/ κ-casein | 9.55 | 2345.06 | | No |
| 2 | TLPFLGAQEQNQ/ κ-casein | 4 | 1473.81 | | No |
| 4 | IIAEKTKIPAVF/β-lactoglobulin | 8.94 | 1477.01 | Antimicrobial (100%) | Yes |
| 4 | VTQTMKGLDIQ/β-lactoglobulin | 6.19 | 1361.77 | | No |
| 4 | KKIIAEKTKIPAVF/β-lactoglobulin | 10 | 1733.39 | Antimicrobial (100%) | Yes |
| 4 | KYLLFCMENS/β-lactoglobulin | 6.32 | 1334.72 | | No |
| 4 | PMHIRLSFNPT/β-lactoglobulin | 10.1 | 1312.71 | | No |

*Anticp analyses: physicochemical characteristics from identified whey-derived peptides; **MBPD analyzes: To identify peptides with bioactivity, the minimum level of similarity selected was 70%. The indicated activity is based on the similarity with the sequences deposited in MBPD; ***iACP analyzes.

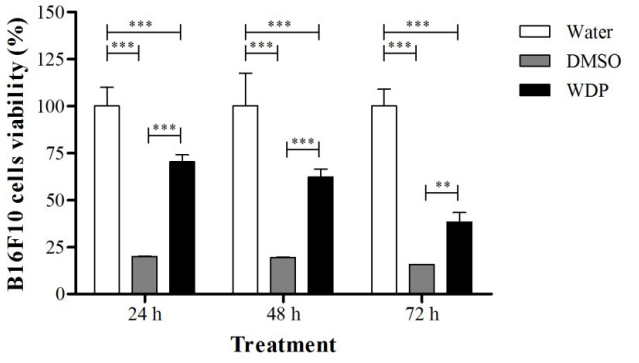


**Figure 1**. B16F10 cell viability after 72 h of treatment with whey-derived peptide (WDP). The cell viability was determined using a resazurin assay, normalizing cell viability of water-treated cells as 100%. All data are expressed as means ± standard deviation and were statistically analyzed by two-way analysis of variance (ANOVA) with Bonferroni posttests. ** p<0.01; *** p<0.001.

tumor cell surface. Ronis et al. (2015) studied the protective effect of whey protein hydrolyzate and whole whey against mammary tumor induction in rats. The rats treated with whey hydrolyzates showed less adenocarcinoma incidence than the other treatments. This result was attributed to bioactive peptides or other substances obtained from whey hydrolyzate-based diet. Ramkisson et al. (2020) showed that *Amaranthus cruentus* hydrolysates share antioxidant effect and also anticancer activity against MCF-7, A549 and HEK 293 cells. Hamin Neto et al. (2019) have already demonstrated the antioxidant effect of whey hydrolyzate generated by metallopeptidase from *E. javanicum*. Here, WDP showed cytotoxic potential and anticancer activity against B16F10 murine melanoma cells. The possibility of multiple biological activities in WDP may culminate in a synergistic action against cancer cells. Therefore, hydrolyzates from bovine milk could be cytotoxic against some tumor cells, including B16F10 melanoma cells, and it could be related to apoptosis or cell cycle arrest. In addition, the cytotoxic selectivity is related to the peptide-cell interaction mediated by electrostatic interactions. The identification of peptides with anticancer properties in the WDP sample (Table 1) also corroborates the results obtained in cell viability studies (Figure 1). For this reason, a proteomic analysis was performed in order to identify proteins involved in these findings.

### *3.3 Melanoma cell (B16F10) proteomic assay: control and WDP treatment*

Treatment with WDP for 72 h reduced the viability of B16F10 cells (Figure 1), and for this reason the subsequent proteomic analyzes were performed under these conditions. The protein profiles were analyzed using mass spectrometry and were determined for differentially abundant proteins between the proteome profile of B16F10 cells subjected to the treatment and the control. In this work, were identified during the WDP treatment 8 proteins significantly upregulated and 6 proteins significantly downregulated as differentially expressed proteins (fold change > 2 and a false-positive discovery rate-adjusted $p < 0.01$) (Table 2 and Table 3).

### *3.4 Upregulated proteins from B16F10 cells treated with whey-derived peptides*

WDP-treatment, compared to the control, increased the abundance of the following proteins: histone H2A type 2-B,

**Table 2**. List of differentially up-regulated proteins from B16F10 melanoma cells treated with whey-derived peptides generated by microbial enzymatic hydrolysis.

| Protein accession[a] | Protein names | fold change[b] | log2(FC) | p value |
|---|---|---|---|---|
| **Q64522** | Histone H2A type 2-B (H2a-613A) | 5.6279 | 2.4926 | 0.0016 |
| **P32020** | Non-specific lipid-transfer protein (NSL-TP) (EC 2.3.1.176) (Propanoyl-CoA C-acyltransferase) (SCP-chi) (SCPX) (Sterol carrier protein 2) (SCP-2) (Sterol carrier protein X) (SCP-X) | 2.5938 | 1.3751 | 0.0029 |
| **Q9JII5** | DAZ-associated protein 1 (Deleted in azoospermia-associated protein 1) | 2.4976 | 1.3205 | 0.0005 |
| **Q9D338** | 39S ribosomal protein L19, mitochondrial (L19mt) (MRP-L19) | 2.4026 | 1.2646 | 0.0017 |
| **P08003** | Protein disulfide-isomerase A4 (EC 5.3.4.1) (Endoplasmic reticulum resident protein 72) (ER protein 72) (ERp-72) (ERp72) | 2.1675 | 1.116 | 0.0073 |
| **P01887** | Beta-2-microglobulin | 2.1197 | 1.0839 | 0.0028 |
| **P0DP28** | Calmodulin-3 | 2.0833 | 1.0589 | 0.0021 |
| **Q60696** | Melanocyte protein PMEL (Melanocyte protein Pmel 17) (Premelanosome protein) (Silver locus protein) [Cleaved into: M-alpha; M-beta] | 2.052 | 1.037 | 0.0045 |

[a]Protein accession code from Uniprot database (The UniProt Consortium, 2019); [b]fold change was determined by treatment abundance compared to untreatment abundance.

**Table 3**. List of differentially down-regulated proteins from B16F10 melanoma cells treated with whey-derived peptides generated by microbial enzymatic hydrolysis.

| Protein accession[a] | Protein names | fold change[b] | log2(FC) | p value |
|---|---|---|---|---|
| **P17182** | Alpha-enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (Enolase 1) (Non-neural enolase) (NNE) | 0.48853 | -1.0335 | 0.0012 |
| **Q9CRB9** | MICOS complex subunit Mic19 (Coiled-coil-helix-coiled-coil-helix domain-containing protein 3) | 0.47626 | -1.0702 | 0.0028 |
| **Q3V3R1** | Monofunctional C1-tetrahydrofolate synthase, mitochondrial (EC 6.3.4.3) (Formyltetrahydrofolate synthetase) | 0.46991 | -1.0896 | 0.0013 |
| **P56480** | ATP synthase subunit beta, mitochondrial (EC 3.6.3.14) (ATP synthase F1 subunit beta) | 0.45166 | -1.1467 | 0.0011 |
| **P05064** | Fructose-bisphosphate aldolase A (EC 4.1.2.13) (Aldolase 1) (Muscle-type aldolase) | 0.41565 | -1.2666 | 0.0011 |
| **Q3U781** | MCG21131, isoform CRA_a | 0.41457 | -1.2703 | 0.0014 |

[a]Protein accession code from Uniprot database (The UniProt Consortium, 2019); [b]fold change was determined by treatment abundance compared to untreatment abundance.

nonspecific lipid-transfer protein (NSL-TP) (EC 2.3.1.176), DAZ-associated protein 1, 39S ribosomal protein L19, protein disulfide-isomerase A4 (EC 5.3.4.1), Beta-2-microglobulin, Calmodulin-3, and melanocyte protein PMEL (Table 2).

Among the upregulated proteins in WDP treatment, some proteins have been shown to decrease tumorigenesis. Beta-2-microglobulin (β2m) is a class I major histocompatibility complex (MHC) protein involved in peptide antigen presentation and β2m downregulation provided that malignant cells continue to grow and metastasize (Garrido et al., 2017). Del Campo et al. (2014) related that metastatic melanoma is able to evade T-cell-mediated immune response by losing of β2m. Therefore, the highest abundance of this protein favored the immune response against cancer cells.

39S ribosomal protein L19, mitochondrial (MRP-L19) was overexpressed in WDP treatment and in stressful conditions, such as drug treatments, some ribosomal proteins could activate the p53 mRNA translation and it induces cell cycle arrest and apoptosis (Xu et al., 2016). Protein disulfide-isomerase A4 (PDIA4) is a protein of the PDI family and some these proteins have modulated tumor growth and chemoresistance in several tumor cell lines (Samanta et al., 2017). WDP treatment also increased calmodulin-3 (CaM-3) and CaM regulates apoptotic processes (Berchtold & Villalobo, 2014). The rupture of homeostasis could trigger responses that provide cell adaptation focused on damage repair, and CaM-3 and PDIA4 could illustrate this attempt to contain apoptosis (Lovat et al., 2008).

Normal signaling pathways could be disturbed by different substances; consequently, mechanisms to reestablish homeostasis are triggered. The effect of WDP treatment in the proliferative assay and the upregulation of stress response proteins suggests that the stressful treatment induces proteins to reestablish cell homeostasis in order to avoid the induction of apoptosis. In this regard, WDP treatment could suppress the proliferation of B16F10 cells and tumor aggressiveness.

### *3.5 Downregulated proteins from B16F10 cells treated with whey-derived peptides*

WDP-treatment reduced the abundance of the following proteins: alpha-enolase, MICOS complex subunit Mic19, monofunctional C1-tetrahydrofolate synthase (mitochondrial), ATP synthase subunit beta (mitochondrial), fructose-bisphosphate aldolase A and MCG21131, isoform CRA_a (Table 3).

Some of these downregulated proteins by WDP treatment have been involved in cancer progression. The abundance of alpha-enolase (ENOA), fructose-bisphosphate aldolase A

(ALDOA) and ATP synthase subunit beta (mitochondrial) (ATP5B) was reduced by WDP-treatment. Melanoma cells MeWo showed that enolase 1 was downregulated by ascorbic acid treatment and proposed this protein as an oncotarget in melanoma (Cecconi et al., 2018). Wang and collaborators observed the downregulation of ALDOA in B16 melanoma cells mediated by fucoidan treatment (Wang et al., 2017), and its depletion reduced the tumorigenic potential (Wang et al., 2018). Bianchi et al. (2018) showed that curcumin inhibited the growth of B16 murine melanoma and suggested that the ATP synthase inhibition caused the energy impairment led to a reduction in cancer cell viability.

WDP treatment decreased the MICOS complex subunit Mic19 (CHCHD3) abundance. Cecconi et al. (2018) showed the downregulation of Mic19/CHCHD3 in melanoma cells MeWo treated with ascorbic acid treatment and the authors related this protein to the apoptosis process.

The protein MCG21131, isoform CRA_a, is a serine - and arginine-rich splicing factor 3 (SRSF3 or SRp20) was previously identified as involved in pre-mRNA alternative splicing regulation (Zhang & Manley, 2013), however, it has been identified in regulation of several cellular functions, RNA splicing, RNA export, RNA translation, RNA polyadenylation, transcriptome and genome integrities, termination of transcription, miRNA process and DNA repair. Therefore, it has been identified in multiple physiological and pathological processes (Zhou et al., 2020) and as an oncogene (Dvinge et al., 2016; Guo et al., 2018).

Here, the abundance of SRSF3 was reduced in B16 melanoma cells submitted to WDP-treatment. The downregulation of SRSF3 in normal human fibroblasts leads to alternative splicing of TP53 and generates p53; and it also suggested that SRSF3 regulates p53 (Tang et al., 2013). Jia et al. (2010) showed that knockdown of SRp20 RNAi-mediated in cancer cells (HeLa and U2OS) resulted in proliferation impairment and apoptosis. Kim et al. (2017) showed that the knockdown of SRSF3 inhibited the proliferation and metastatic potential of osteosarcoma U2OS cells. Similar results were also observed in colon cancer cells and proposed that SRSF3 is involved in G1-phase progression and apoptosis regulation (Kurokawa et al., 2014). He et al. (2011) studied ovarian cancer cells, and knockdown of SRp20 also triggered apoptosis in these cells. Lu et al. (2014) showed that tumor suppression by caffeine treatment was modulated via the alternative splicing of the target genes of SRSF3.

Enhanced glycolysis and other changes in energy metabolism are needed for cancer cell proliferation (Li & Zhang, 2016). Thus, the downregulation of ENOA, ALDOA, and ATP5B could impair melanoma proliferation due to a reduction in energetic routes. Interestingly, the bioactive peptides provided by the WDP-treatment also affected the abundance of the well-known oncogene SRSF3/SRp20 with a reduction closely related to apoptosis induction in tumor cells.

## 4 Conclusions

Previous study showed the ability of metallopeptidase from *E. javanicum* to produce WDP with bioactivity. In this study, we described that this metallopeptidase also released WDP that were able to impair B16F10 cell line viability, as well as modulated important proteins related to cancer cell progression and survival. Therefore, WDP exhibits the characteristics of a potential chemopreventive drug candidate against melanoma.

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