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Antioxidant and antimicrobial properties of an extract rich in proteins obtained from *Trametes versicolor*

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Abstract

Introduction: Bioactive proteins and peptides generated from fruit, vegetables, meat or fish have great potential as functional food or substitutes for antibiotics. In recent years it has also been demonstrated that the fungus kingdom could be a source of these compounds. The study investigated the bioactivity of an extract of the lignicolous fungus *Trametes versicolor* and its hydrolysate. **Material and Methods:** The fungus was collected in a mixed forest in October, extracted and hydrolysed. To inspect the protein and peptide profiles before and after hydrolysis, matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometric analysis was performed. To evaluate the antioxidant properties of the preparations, 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS$^{\bullet+}$) radical scavenging assays were used. The activity of the fungus extract and hydrolysate against *Aeromonas veronii*, *Bacillus cereus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* was determined by the minimum inhibitory concentration and minimum bactericidal concentration values. **Results:** The extract and its hydrolysate showed almost 100% ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ radical scavenging with a low half maximal inhibitory concentration. The water extract and hydrolysate of *T. versicolor* exhibited antimicrobial activity against two *S. aureus* strains, *E. coli*, *P. aeruginosa* and *Salmonella* Typhimurium. **Conclusion:** These results provide compelling evidence that the analysed fungus extract and its hydrolysate hold promise with their antibacterial and antioxidant properties.

Keywords: bioactive proteins, bioactive peptides, *Trametes versicolor*, antioxidant properties, antibacterial properties.

Introduction

The latest research emphasises natural alternatives to the standard methods of food preservation, highly nutritious functional foods and substitutes for antibiotics. Those health-enhancing substances may owe their effects to biologically active proteins. Furthermore, proteins can also be a source of bioactive peptides obtained through protease activity. Bioactive peptides are short residues containing from 2 to 30 amino acids and can be extracted from various food sources such as fruit, vegetables, meat or fish (28). These kinds of compounds have a physiological significance beyond their average nutritional value. Currently, there is a growing interest in the fungus kingdom because bioactive peptides may also be obtained from fungi. Studies of these organisms have indicated the presence of a significant protein content that often exceeds those of vegetables and fruit (32). *Trametes versicolor* is a common fungus species from the *Polyporaceae* family found throughout the world. The fruiting bodies of *T. versicolor* can reach up to 10 cm in diameter and are

most often found in clusters on tree trunks. The top layer of its fruiting body is varicoloured in concentric bands, while the tubular hymenophore is from creamy to light yellow in colour. This mushroom has been used in folk medicine for thousands of years, while more recently it has been exploited as a source of polysaccharide K as an adjuvant immunotherapy for a variety of cancers (10).

The aim of the study was to investigate the bioactivity of *Trametes versicolor* extract (TVE) and its hydrolysate (TVH). In this particular study, the bioactivity of a protein-rich extract from *T. versicolor* and the tryptic hydrolysate was evaluated. This organism was chosen because of its lack of toxicity to living organisms.

Material and Methods

Optimisation of fruiting body extraction. The fruiting bodies of *T. versicolor* were harvested in October (average temperature 15°C) in a mixed forest. Next, they were thoroughly washed to remove any residue of tree bark, and the connection point between the fruiting body and the bark was removed. The cleansed fruiting bodies were cut into small pieces and stored overnight at −80°C. After ultra-low temperature freezing, the material was immersed in liquid nitrogen in a porcelain crucible for 5 min, and subsequently the fruiting bodies were mashed into a powder. Liquid extraction was conducted using Milli-Q water and 50 Mmol tris(hydroxymethyl)aminomethane (tris)–HCl buffer. In order to find the conditions resulting in the highest amount of protein and peptide, extraction was undertaken at three temperatures: 30°C, 60°C and 80°C. For this purpose 5 g of *T. versicolor* powder was placed in 100 mL of water or tris buffer. Only a small amount of the *T. versicolor* was used because of its tubular hymenophore, which affected the swelling of the fungus during immersion in water. Each extraction lasted for 3 h, which was the time required for two steady protein concentrations to be obtained. The obtained mixtures were centrifuged twice for 30 min at 5000 × *g*, and the resulting supernatants were divided into portions and stored at −80°C.

Protein hydrolysis. The obtained extract was subjected to hydrolysis by tryptic digestion in order to obtain mixtures with a higher amount of peptides and to establish what improvement in properties there was in comparison with the initial mixture. For this purpose the extract with the highest protein content was heated to 40°C with the addition of $CaCl_2$. Trypsin was added after the pH value was adjusted to 8.0. The amount of enzyme was strictly correlated with the amount of protein and was found to be 5, 10 and 20% of the protein concentration. The reactions were carried out until a constant concentration of peptides in the hydrolysates was attained. Finally, enzyme inactivation was conducted at 100°C for 10 min. The degree of hydrolysis was determined by measuring the quantity of the soluble peptides. A 500 µL volume of hydrolysate was collected at intervals and mixed immediately with 500 µL of 10% trichloroacetic acid. After 15 min of incubation, the sample was centrifuged for 10 min at 5500 × *g*. The content of soluble peptides in the supernatant was determined using a NanoMaestro spectrophotometer (MaestroGen, Hsinchu City, Taiwan) by measuring the absorbance at 280 nm. The degree of hydrolysis was calculated as the solubilised peptide as a percentage of the total protein content.

Determination of free radical scavenging by assay. All of the tested samples were subjected to antioxidant activity tests employing a 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical and a 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical cation. Scavenging of $DPPH^{\bullet}$ radicals was determined using a modified Brand-Williams assay (9). For the purpose of this test, 0.5 mM of $DPPH^{\bullet}$ radical solution was diluted by ethanol to a 0.9 absorbance value at 517 nm. Colour change was caused by addition of 1 mL of fungus extract or hydrolysate mixed with 1 mL of ethanol and 1 mL of $DPPH^{\bullet}$ radical solution. The absorbance (Abs) measurements were made after 30 min of incubation in a dark place. The percentage of inhibition was calculated as follows:

$$\%\ DPPH^{\bullet}\ \text{radical inhibition} = ((Abs_{control} - Abs_{sample})/Abs_{control}) \times 100$$

The $ABTS^{\bullet+}$ radical scavenging assay was conducted according to Nenandis *et al.* (23). A mixture of potassium persulfate ($K_2S_2O_8$) and $ABTS^{\bullet+}$ radicals was placed in the darkness for an overnight incubation. The next day, the $ABTS^{\bullet+}$ radical mixture was diluted with phosphate-buffered saline until the absorbance value at 734 nm was equal to 0.7. A 1 mL volume of the prepared $ABTS^{\bullet+}$ cation radical solution was mixed with 50 µL of fungus extract or hydrolysate in a test tube. The mixture was vortexed and transferred into a semi-micro cuvette. After 6 min in the darkness, the mixtures were placed in the spectrophotometer and the decrease of absorbance at 734 nm was measured. The percentage of inhibition was calculated as follows:

$$\%\ ABTS^{\bullet+}\ \text{radical inhibition} = ((Abs_{control} - Abs_{sample})/Abs_{control}) \times 100$$

For both reactions, the antioxidant capacities of the fungus samples were described as the percentage decrease in colour intensity compared with the blank control sample without fungus mixtures. Additionally, as part of the comparison, the antioxidant capacity of lyophilised fruits commonly considered to be rich sources of antioxidants was tested. The concentration range for fungus proteins and peptide samples in $DPPH^{\bullet}$ was 0.25–8.00 mg/mL and in $ABTS^{\bullet+}$ was 0.16–20.00 mg/mL. For vegetables and fruits it was 0.25−5.00 mg/mL and 0.50−5.00 mg/mL, respectively.

Matrix-assisted laser desorption/ionisation–time-of-flight (MALDI-TOF) mass spectrometry for protein and peptide profiling of fungus samples. In order to check the protein and peptide profiles before and after hydrolysis, mass spectra were measured in three mass ranges: 700−4,000 Da, 5,000−10,000 Da and

10,000−20,000 Da. For this purpose fungus samples were purified and concentrated using ZipTip 0.2 µL pipette tips with C18 resin (Merck, Darmstadt, Germany) for acetonitrile/0.1% trifluoroacetic acid elution according to the manufacturer's protocols. A 1 µL volume of the obtained and purified sample was spotted onto an AnchorChip MALDI plate (Bruker Daltonics, Bremen, Germany). Next, the dried sample spots were covered by 1 µL of a saturated α-cyano-4-hydroxycinnamic acid matrix solution. The mass spectra were recorded in positive reflector mode using an UltrafleXtreme MALDI tandem time-of-flight spectrometer (Bruker) and flexControl 3.3 (Bruker) software. They were smoothed using the Savitzky–Golay method, the baseline was corrected with the top-hat baseline algorithm, and a list of peaks for a signal-to-noise ratio >3 was generated using flexAnalysis 3.4 software (Bruker).

Antimicrobial activity. The antimicrobial activity of the tested extract and hydrolysate was evaluated against two panels. One assembled reference microorganisms from both the American Type Culture Collection (ATCC) and the German Collection of Microorganisms and Cell Cultures (DSMZ) and comprised Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, methicillin-resistant *Staphylococcus aureus* ATCC 1707, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* ATCC 19434 and *Bacillus cereus* ATCC 10876) and Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 14028, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853 and *Aeromonas veronii* DSM 7386). The second included clinical isolates (*Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*). The microbial suspensions with fresh 24-h cultures in sterile 0.85% NaCl were evaluated using the 0.5 McFarland turbidity standard and then 0.1 mL of the suspension was diluted 100-fold in 9.9 mL of sterile Mueller–Hinton broth (Biomaxima, Lublin, Poland) (for aerobic bacteria) or Mueller–Hinton broth +5% defibrinated horse blood and 20 mg/L β-nicotinamide adenine dinucleotide (for streptococci) to obtain a final density of 1.5×10^6 colony-forming units (CFU)/mL.

Minimum inhibitory concentration (MIC) assays. The studied extracts were screened for antibacterial activities using a microdilution broth method according to the protocols of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The sterile 96-well polystyrene microtitre plates (Medlab, Raszyn, Poland) were prepared by adding to each well 100 µL of the extract and hydrolysate tested, which had been serially diluted in an appropriate broth medium in order to obtain final concentrations of the tested extract and hydrolysate ranging from 0.01563 to 32 mg/mL. After overnight incubation at 35 ± 2°C, the MICs were determined visually, using an optically clear well as a criterion, and also using an ELx800 microplate reader (Bio-Tek, Winooski, VT, USA) for spectrophotometric absorbance at 570 nm. The appropriate controls were included on each microplate, the positive one containing the inoculum of all of the microbial strains without the tested compounds and the negative one containing the tested extract and hydrolysate without the inoculum and including a sterile broth medium.

Minimum bactericidal concentration (MBC) assay. A 5 µL volume was taken from the well determined in the MIC experiment to be the one presenting the MIC value and also from the two wells above the MIC value well and these were plated on the recommended Mueller–Hinton agar. All of the plates were incubated for 24 h at 35 ± 2°C under aerobic conditions for all microorganisms. The MBC values were defined as the lowest concentrations of the extract and hydrolysate without any visually apparent growth of microorganisms (no bacterial growth in colony form observed). All of the measurements were performed at least in triplicate. The MBC:MIC ratio was estimated in order to investigate the bactericidal or bacteriostatic effects of the extracts tested. The experiments were repeated in triplicate.

Results

The content of basic nutritive components of the fungus was measured and the results are shown in Table 1, and the content of fatty acids in *Trametes versicolor* and animal-based products are presented in Table 2. The tested mushrooms had a high content of monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and a relatively low content of saturated fatty acids (SFA).

Table 1. Nutritional values of *T. versicolor*

Parameter	*Trametes versicolor*
Crude protein g/100g	5.50
Calorific value kJ/100g	491.00
Moisture g/100g	45.64
Ash g/100g	1.23
Crude lipids g/100g	0.43
Crude fibre g/100g	46.73
Sodium chloride %	0.01

Table 2. Content of fatty acids in *Trametes versicolor* and animal-based products according to the National Agricultural Library Digital Collections (1)

Material	SFA	MUFA	PUFA
Trametes versicolor	34.7	23.7	22.7
Turkey fat	29.4	42.9	23.1
Butter	50.5	23.4	3.0
Lard	39.2	45.1	11.0
Beef tallow	49.8	41.8	4.0

SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids

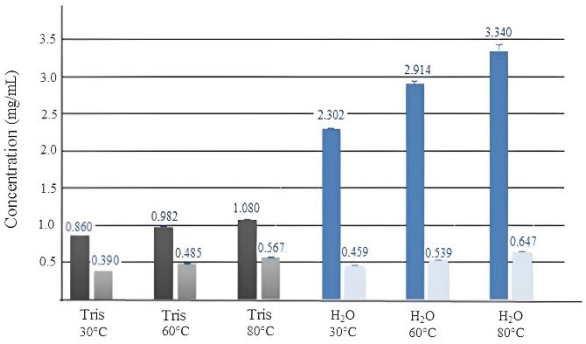

Fig. 1. The efficiency of protein and peptide extraction from *Trametes versicolor* using water or tris(hydroxymethyl)aminomethane (Tris) buffer at different temperatures

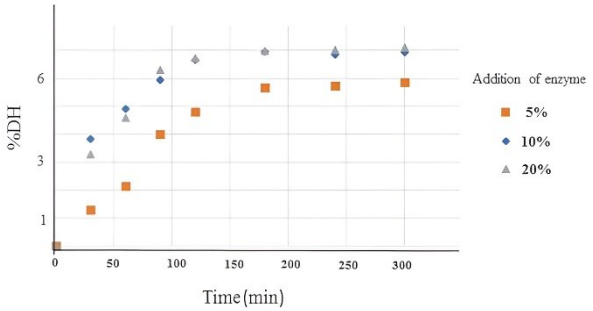

Fig. 2. Degree of tryptic hydrolysis for *Trametes versicolor*
DH – degree of hydrolysis

Table 3. Half maximal inhibitory concentration (IC_{50}) values for radical scavenging by fungus samples and common fruit and vegetables

Sample	IC_{50} $ABTS^{\bullet+}$ (mg/mL)	IC_{50} $DPPH^{\bullet}$ (mg/mL)
TVE	2.25	6.30
TVH	1.94	1.75
Blueberry	1.19	0.27
Blackcurrant	2.78	0.39
Spinach	4.30	3.50
Apple	4.62	1.29

$ABTS^{\bullet+}$ – 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; $DPPH^{\bullet}$ – 2,2-diphenyl-1-picrylhydrazyl; TVE – *Trametes versicolor* extract; TVH – *Trametes versicolor* hydrolysate

The highest increments in the concentrations of TVE and TVH in their solvents were observed during the first 30 min of extraction. The extraction efficiency results are presented in Fig. 1. Among all of the reactions, water extraction at 80°C showed the highest protein and peptide extraction yield, and generally water gave a significantly higher yield than that achieved by the tris-HCl buffer (*t*-test, $P < 0.05$).

The degrees of enzymatic hydrolysis of the *T. versicolor* extract achieved after tryptic digestion are presented in Fig. 2. The degree of hydrolysis (DH), which may be understood as the number of broken peptide bonds in relation to the original protein, was highest for the 10% and 20% additions of the enzyme. The hydrolysis of fungus proteins increased rapidly for the first hour of incubation, with the enzyme finally reaching hydrolysis equilibrium at around the end of the second hour of the process. The high rate of reaction in the first minutes was due to the hydrolysis of the peripheral section of the proteins. The similarity in peptide concentrations between the 10% and 20% trypsin addition indicated that there was no need to use a higher amount of protease. The increased temperature and the specific amino acid composition of the hydrolysed samples may be responsible for the satisfactory efficiency of the reaction; it is established that an elevated temperature during hydrolysis can result in conformational changes. These changes may more fully expose strategic peptide bonds, thereby allowing the enzyme better access.

In the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging assays, all of the tested samples showed a significant percentage of radical inhibition, and it was shown that *T. versicolor* hydrolysate possessed stronger antioxidant properties. The chosen concentrations of the mixtures used for testing the antioxidant properties were based on their solubility. The results of radical scavenging are presented in Fig. 3. The solution of protein extract was capable of inhibiting approximately 60% of both radicals. The tryptic hydrolysate, in turn, reached 94% inhibition of the $ABTS^{\bullet+}$ radical. A slightly lower proportion of inhibition was observed for the $DPPH^{\bullet}$ radical, which was 86%. Also the IC_{50} value was calculated, which is the concentration of an antioxidant-containing mixture at which it scavenges 50% of the initial radicals (Table 3).

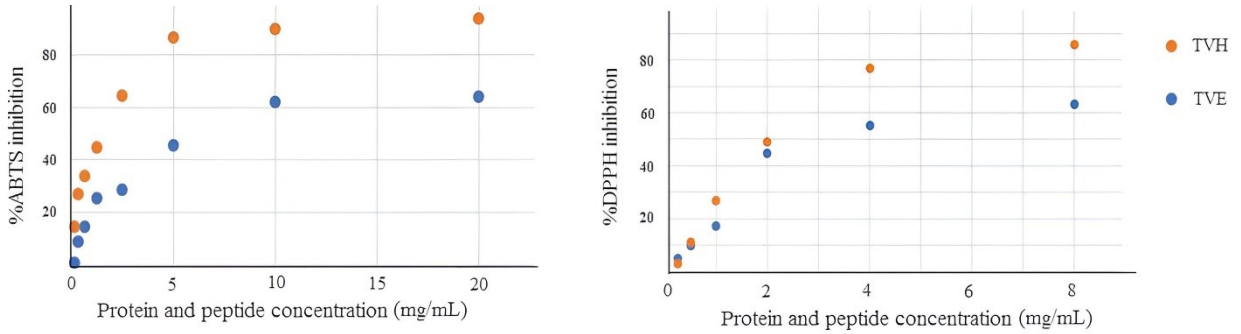

Fig. 3. 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) scavenging activity of *T. versicolor* extract (TVE) and its hydrolysate (TVH)

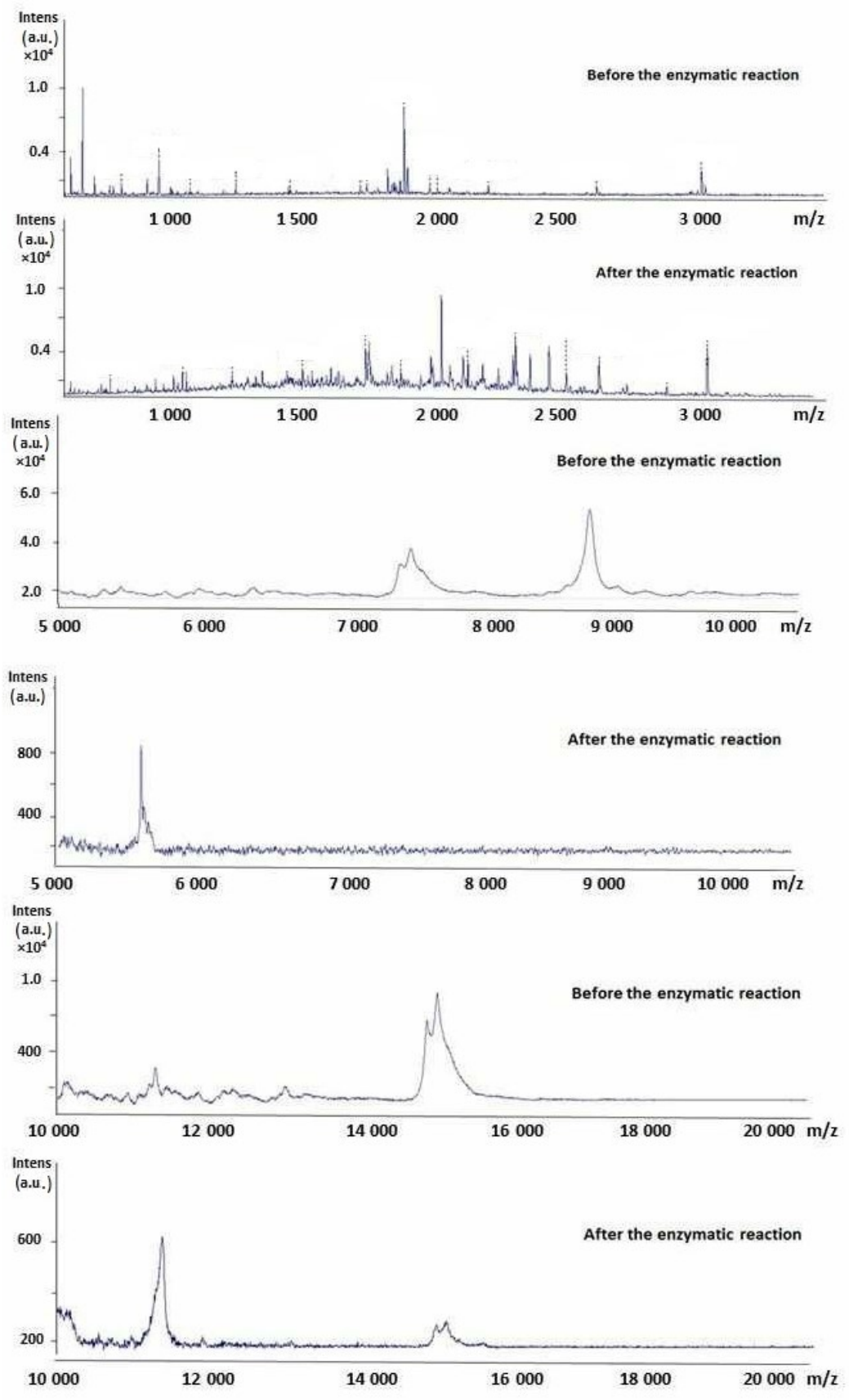

Fig. 4. Mass spectra obtained before and after enzymatic hydrolysis of a high-protein extract of *Trametes versicolor*
a.u. – arbitrary units

Table 4. Antibacterial activity of tested extracts and hydrolysate

Strains			MIC, MBC values (μg/mL) (MBC:MIC)	
			TVE	TVH
Reference	Gram +	*Staphylococcus aureus* ATCC 6538	62.5, 62.5 (1)	
		Staphylococcus epidermidis ATCC 12228	>	8,000; >
		Staphylococcus aureus ATCC 1707 MRSA	500, 500 (1)	>
		Enterococcus faecium ATCC 19434	>	>
		Enterococcus faecalis ATCC 29212	>	>
	Gram −	*Bacillus cereus* ATCC 10876	8,000, >	>
		Escherichia coli ATCC 25922	4,000, 4,000 (1)	>
		Pseudomonas aeruginosa ATCC 27853	4,000, 8,000 (2)	>
		Salmonella Typhimurium ATCC 14028	4,000, 8,000 (2)	>
		Klebsiella pneumoniae ATCC 13883	>	>
		Aeromonas veronii DSM 7386	>	>
Clinical		*Streptococcus agalactiae*	250, 32,000 (>4)	>
		Streptococcus uberis	250, 32,000 (>4)	>
		Streptococcus dysgalactiae	250, 32,000 (>4)	>
		Staphylococcus aureus	250, 500 (2)	>

MIC – minimum inhibitory concentration; MBC – minimum bactericidal concentration; TVE – *Trametes versicolor* extract; TVH – *Trametes versicolor* hydrolysate

The IC_{50} values for DPPH$^{\bullet}$ were as follows: 2.52 mg/mL for the extract and 1.94 mg/mL for the hydrolysate. For ABTS$^{\bullet+}$, the IC_{50} values were as follows: 6.30 mg/mL for the extract and 1.75 mg/mL for the hydrolysate. It may be clearly observed that the mass of a peptide has an impact on its free radical scavenging, lower mass being more effective. A close relationship between mass and effectiveness was observed for the ABTS$^{\bullet+}$ radical cation. In this case, the hydrolysate had an IC_{50} value more than three times higher than that of the initial extract. The difference was not so visible in the case of the DPPH$^{\bullet}$ radical.

The IC_{50} results obtained for the fungus extract and hydrolysate were compared with the values obtained for popular and frequently consumed fruit and vegetables. The obtained IC_{50} values for the hydrolysates scavenging the two radicals were similar to those of the fruit and vegetables commonly considered to be valuable sources of antioxidants. The results obtained are shown in the table below.

The analysis of the mass spectra for *T. versicolor* revealed differences, especially in the range of higher peptide masses and for small proteins (5,000−10,000 Da and 10,000−20,000 Da) (Fig. 4).

The susceptibility of selected pathogenic microorganisms to TVE as indicated by their MIC and MBC values is shown in Table 4. The extract of *T. versicolor* showed the best activity against methicillin-resistant *S. aureus* ATCC 1707 and *S. aureus* ATCC6538, with respective MIC values of 500 μg/mL and 62.5 μg/mL, and against *Streptococcus uberis*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* with MIC values of 250 μg/mL. Moreover, TVE also showed additional moderate activity (MIC = 4,000 μg/mL) against Gram-negative bacteria (*E. coli*, *P. aeruginosa* and *Salmonella* Typhimurium). In addition, TVH demonstrated favourable activity against Gram-positive *S. epidermidis* (MIC = 8,000 μg/mL). A ratio of MBC to MIC <4, which implied the existence of bactericidal effect, was calculated for TVE against all these bacteria except for the clinical isolates. The fungus extract was noted to be bacteriostatic (MBC:MIC >4) against the clinical isolates of *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae.*

Discussion

Through analysing the nutritional composition of the fungus fruiting body, a high protein content may be observed. Considering the dry weight, the percentage of proteins in the fruiting bodies of *T. versicolor* was 10.19%. This value was promising in terms of using *T. versicolor* as a protein source and for obtaining protein hydrolysates, and furthermore, it indicated that the selected mushroom species has a higher amount of crude protein than many common vegetables (2). According to Zhou *et al.* (32), who analysed 130 fungus samples in research on mushroom-derived bioactive peptides, the average protein content in the dry weight was 23.80 ± 9.82 g/100 g.

By analysing the results of the amount of protein found in the *T. versicolor* fruiting body and the concentration of proteins obtained *via* extraction, the yield of the reaction with the maximal extraction concentration can be calculated as 65%. The reason for the low process efficiency may be the specific tissue structure. Plant- and fungi-based solid samples have a capillary and porous structure which has a significant limiting effect on internal mass movement (4). The specific cell framework is the reason that total diffusion resistance is encountered predominantly in the cell membranes and mass movement takes place through them. The tortuosity of the pores lengthens the diffusion

route and in addition, the collisions of the diffusing particles with the pore walls cause an increase in mass resistance to motion. For this reason, diffusion in solids is lower than in liquids. Low extraction efficiency also can be caused by the physicochemical properties of the extractant. It is principally the hydrophilic nature of tris-HCl that causes the extraction of water-soluble proteins (20). Extraction efficiency is also closely connected with the temperature of the process: for the most part a higher temperature resulted in a higher extraction yield. A rising temperature favours diffusion phenomena by changing the size of the diffusion coefficient. In the case of *T. versicolor*, the amount of extracted proteins and peptides increased with increasing temperature. Among all of the reactions, water extraction at 80°C showed the highest protein and peptide extraction yield. Despite the lower yield of extraction in water at a lower temperature than 80°C, 60°C was chosen as the water temperature for further extraction procedures. This was due to the possibility of higher mass protein instability at 80°C. An analysis of the results indicated that two hours was a sufficient period to obtain the maximum saturation of protein and peptides. After this time, no significant changes in extracted protein or peptide concentrations were noted for either extractant at any temperature. The next step in the experiment was to obtain bioactive peptides by performing trypsin hydrolysis. For trypsin, the appropriate cleave sites are between the carboxyl group of arginine or lysine. According to Simpson *et al.* (26), the rate of cutting is slower when the lysine and arginine residues are connected to acidic amino acids or cysteine. As reported by Angelova *et al*. (6), *T. versicolor* is a fungus rich in arginine, the content of which is 8.0% of all of the amino acids. This makes it the fourth most abundant amino acid in *T. versicolor*. On the other hand, the amount of lysine is much lower, comprising only 3.0%, which is only the fourteenth-ranked amino acid in *T. versicolor*'s composition. The notable degree of hydrolysis that was achieved in a short time may also be due to the small amount of cysteine, which is the amino acid found least often in the fungus.

It may not be possible to achieve better hydrolysis reaction efficiency because *T. versicolor* is in the Basidiomycota division of the fungus kingdom and trypsin inhibitors are known to be present in *basidiomycetes* fungi tissues (27). To date, only a low number of research papers relating to the hydrolysis of fungal proteins have been published. One of the studies dealing with this issue is an article by Ang *et al.* (5), which considers the usefulness of fungal peptides obtained through bromelain hydrolysis. Bromelain was used to cut proteins from such mushrooms as shitake, oyster mushroom, bunashimeji and enoki in order to create the most highly flavoured powder for culinary purposes. The authors presented degrees of hydrolysis which varied from 54 to 67%. Another study that focused on the attainment of fungi hydrolysate is the publication by Palupi *et al.* (24). In this study, a 0.1% (w/w) solution of Protamex protease was the source of paddy straw mushroom (*Volvariella volvacea*) peptides. The reaction was carried out at three different temperatures with different incubation times, achieving hydrolysis which reached 25%. The effectiveness of trypsin, proteinase K and pepsin digestion in three different concentrations of enzymes, temperatures (30°C, 40°C and 50°C), and durations of the hydrolysis (60, 90 and 120 min) was studied by Goswami *et al.* (18). The material used for testing was the dried fruiting body of *Pleurotus ostreatus* grown on paddy straw. The presented results of optimisation clearly showed that in terms of the DH value, the most suitable reaction used 0.15% proteinase K at 50°C for 2 h. The product of protein hydrolysis was characterised by improved foaming stability and emulsifying features (16). Similar DH values after tryptic digestion were achieved by Farzaneh *et al.* (13) for fungal matter from *Agaricus bisporus* and *Terfezia claveryi*. In their findings, the DH value ranged from 7.5 to 20.1%, and the testing samples differed by being or not being blanched. Another confirmation of effective protein extraction and hydrolysis were MALDI-TOF mass spectra, analysis of which confirmed the protein digestion. During hydrolysis, signals at approximately 15,000 Da diminished after the enzymatic reaction. According to the UniProt resource, those masses may be assigned to the phosphoenolpyruvate/pyruvate domain–containing protein, the RNA recognition motif domain–containing protein, the velvet domain–containing protein or the Cauli_VI domain–containing protein. Also, smaller masses of around 7,500 Da and 8,500 Da were digested into lower-mass peptide fragments. The smallest changes in the mass profile were observed in the range of 700–4,000 Da. Small changes in both cases may have been due to the lower possibility of tryptic digestion in shorter amino acid sequences. Additionally, it is most likely that the digestion caused the formation of lower-mass peptides like di- and tripeptide. The molecular masses of this type of compound are not suitable for mass-to-charge ratio scanning in mass spectrometers equipped with a MALDI analyser. Matrix fragments and their adductions may disrupt sample signals causing errors in the determination of sample composition. The obtained mass spectra clearly indicated changes in mass distribution after enzymatic hydrolysis with trypsin. The disappearance of the signal corresponding to the masses in the range of proteins proved the effectiveness of tryptic digestion. Additionally, the mass spectrometry results corresponded with peptide concentration values which increased after the enzymatic reaction.

The lower value of IC_{50} for the hydrolysate was mainly caused by the presence of bioactive peptides. There are two major routes to obtain these compounds. The first one is protein precursor degradation *via* enzymatic reaction, microbial fermentation or chemical degradation (exogenous peptides), and the second one is active production in the organisms (endogenous peptides) (3). These short fragments contain up to 20 amino acids, and unlike the proteins from which they

are released, bioactive peptides can show specific bioactivities including the scavenging of free radicals. Significant antioxidant properties may also originate from the amino acid composition of the tested fungus. According to Dong *et al*. (12), processes that take place *via* electron donation make them stable and inactive. Another factor affecting the antioxidant properties of a compound is the presence of hydrophobic amino acids like histidine, cysteine and methionine. The mechanism of this activity is connected with improvements in peptide solubility in the lipid environment and in facilitating their availability to nonpolar radicals (25). The amino acid composition of *T. versicolor* was analysed and showed a high content of histidine (6). The high degree of scavenging of radicals may be due to a protonated form of the imidazole ring (21). Positively charged histidine is able to function as a proton donor for active free radicals like $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, which must assume a reduced form in order to become inactivated. Histidine is the main amino acid in the composition of *T. versicolor*. Fungus extract may be rich in many proven antioxidant substances, such as polysaccharides or phenols (7, 29). In the present study, the better results for the hydrolysate than for the initial extract clearly showed that there is a relationship between antioxidant properties and peptide composition. At present, there are few reports confirming the radical scavenging ability of fungus proteins and their hydrolysates. Dong *et al.* (11) analysed a peptide fraction with a mass below 3 kDa obtained from *Grifola frondosa* through trypsin digestion and produced results similar to those of the present study. This low-mass fraction achieved 89.6% $DPPH^{\bullet}$ radical removal when concentrated at 2.5mg/mL. In another study, the scavenging of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals by a *Morchella esculenta* mixture obtained *via* simple and environmentally friendly microwave irradiation was tested. In this case, the radicals were successfully blocked by hydrogen-donating antioxidants such as peptides under the appropriate environmental conditions. The IC_{50} values observed were 6.03 mg/mL for $DPPH^{\bullet}$ and 0.071 mg/mL for $ABTS^{\bullet+}$ (30). Another example of fungus peptide extracts with antioxidant properties are those obtained from the *Ganoderma lucidum* fruiting body. In a paper investigating this fungus, the peptides were obtained by Sephadex G-25 and Sephadex G-50 purification and a reverse-phase high-performance liquid chromatography method. Scavenging of the $DPPH^{\bullet}$ radical reached 74.21% efficiency (17).

The antibacterial activity of the extract and hydrolysate of *T. versicolor* were tested against a wide range of pathogenic microorganisms, namely *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecium, Enterococcus faecalis, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Salmonella* Typhimurium, *Klebsiella pneumoniae* and *Aeromonas veronii*. The extract and hydrolysate of *T. versicolor* showed antibacterial effects on the growth of the cultures of staphylococcal and enterococcal reference strains. Values of MIC less than 32,000 µg/mL for the extract or hydrolysate were considered to be highly active. The results are consistent with those of Chowdhury *et al.* (11), who showed MIC values of 6,000 µg/mL and 7,000 µg/mL of a methanolic extract of *Pleurotus ostreatus* for *S. aureus* and *B. subtilis*, respectively. They are also in agreement with the data of Gashaw *et al.* (15), who reported the activity of a methanolic extract of *Pleurotus ostreatus* mushrooms against *E. faecalis* and *B. subtilis* with an MIC value of 7,000 µg/mL. *Trametes versicolor* extract was found to be the most effective against staphylococci with its bactericidal activity and against *B. cereus* with its bacteriostatic activity. This is similar to the findings of Matijašević *et al.* (22) for *T. versicolor* (also called *Coriolus versicolor*), who reported higher MIC values of 62.5–2,500 µg/mL and 5,000 µg/mL for staphylococci and *B. cereus*, respectively. In a similar experiment (16), chloroform and 70% ethanol extracts of *T. versicolor* were found to have MIC values of 830–1,170 µg/mL against *S. aureus* ATCC 25923 and methicillin-resistant *S. aureus* ATCC 33591. The most promising *in vitro* antibacterial activity against Gram-negative bacteria was shown by TVE, which was bactericidal against Gram-negative rods and had an MBC:MIC ratio no greater than 2. This is in agreement with Matijašević *et al.* (22), who demonstrated higher MIC values of 5,000–20,000 µg/mL of *T. versicolor* 10% methanol extract against *Yersinia enterocolitica*, *Shigella sonnei*, *Salmonella enteritidis*, *E. coli* O157:H7 and *Proteus hauseri*. Higher MIC values of 1,000–1,330 µg/mL were reported by Gebreyohannes *et al.* (16) for chloroform and 70% ethanol extracts of *T. versicolor*, which were bactericidal against *K. pneumoniae* ATCC 13883 and clinical isolates of *E. coli* and *P. aeruginosa*. The extract and hydrolysate of *T. versicolor* showed differential antibacterial effects on the growth of cultures of clinical streptococcal isolates. The lowest MIC value of 62.5 µg/mL was observed against all clinical streptococcal isolates for the *T. versicolor* extract which also showed bacteriostatic activity.

Trametes versicolor demonstrated the investigated activities to extents which suggest the benefit of this fungus, but there is more to recommend it. The negligible fat and salt contents and low calorific value of *T. versicolor* largely obviate the need to impose restrictions on consumption of food based on *T. versicolor*. The fatty acid profile of fungi including *T. versicolor* is also a positive characteristic. A comparison of the values for SFA, MUFA and PUFA with those of common animal food sources made evident that fungus samples had a smaller amount of SFA. Saturated fatty acids are a key thread in the hypothesis which links diet to coronary heart disease risk, so it is essential to eliminate this kind of compounds from the everyday diet. Fatty acids belonging to the MUFA and PUFA groups are important for the preservation of health. The revealed content of MUFA and PUFA in *T. versicolor* can be defined as significant. In addition, according to the

literature mixtures rich in peptides may show bioactivity in multiple ways, besides antioxidant and antibacterial properties. Angiotensin-converting enzyme inhibitory or opioid activities have also been attributed to these mixtures (14, 19, 31). Peptides can also demonstrate very favourable bioavailability and for that reason they are often used in functional food and dietary supplement industry applications.

In conclusion, the obtained protein-rich extract and its hydrolysate are promising solutions with antibacterial and antioxidant properties. The hydrolysate of *T. versicolor* showed a high percentage of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging, which reached almost 100%, and exerted its effect at a low IC_{50} value. After further research, the tested solution may be used in the future as a natural preservative or anti-aging supplement because of its antioxidant properties. By analysing MIC and MBC values we may conclude that water extract and hydrolysate from *T. versicolor* have the potential to be interesting alternatives to antibiotics due to their antimicrobial activity against bacteria strains such as *S. aureus* ATCC 6538, *S. aureus* ATCC 1707 MRSA, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S.* Typhimurium ATCC 14028. Moreover, the elimination of harmful extractants such as organic solvents, and their replacement with hot water creates the possibility of using the designed mixtures in the food or pharmaceutical industries.

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