

The use of serine protease from *Yarrowia lipolytica* yeast in the production of biopeptides from denatured egg white proteins

Marta Pokora¹, Aleksandra Zambrowicz¹✉, Agnieszka Zabłocka², Anna Dąbrowska¹, Marek Sołtyś¹, Konrad Babij¹, Ewelina Eckert³, Tadeusz Trziszka¹ and Józefa Chrzanowska¹

¹Department of Animal Products Technology and Quality Management, Wrocław University of Environmental and Life Sciences, Wrocław, Poland; ²Department of Immunochemistry, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland; ³Department of Agricultural, Food and Nutritional Science, University of Alberta, 410 Agriculture/ Forestry Centre, Edmonton, AB T6G 2P5, Canada

Deriving non-conventional enzymes from cheaper sources than those used for commercially available enzymes may result in the production of hydrolysates with beneficial features, while drastically reducing the cost of hydrolysis. This is especially significant for enzymatic hydrolysis as a method of protein waste utilization. We have previously described the ability of non-commercial serine protease from *Yarrowia lipolytica* yeast to produce/release bioactive peptides from egg white protein by-products (EP). The enzymatic hydrolysis of EP was carried out for 24 h using the serine protease at an enzyme: substrate ratio of 1:30 (w/w). The obtained hydrolysate was characterized by protein degradation of 38% and also exhibited an antioxidant and cytokine-inducing activity. The isolation procedure (ultrafiltration and RP-HPLC) of bioactive peptides from the EP hydrolysate provided peptide fractions with significant antioxidant and ACE inhibitory activities. Three homogeneous and three heterogeneous peptide fractions were identified using MALDI-TOF/MS and the Mascot Search Results database. The peptides, mainly derived from ovalbumin, were composed of 2-19 amino-acid residues. We have thus demonstrated a novel ability of serine protease from *Y. lipolytica* to release biopeptides from an EP by-product.

Key words: *Yarrowia lipolytica*, serine protease, bioactive peptide, antioxidant, ACE-inhibitor

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✉ e-mail: aleksandra.zambrowicz@up.wroc.pl

Abbreviations: ANOVA, analysis of variance; ACE, angiotensin converting enzyme; AEP, alkaline extracellular protease; DH, degree of hydrolysis; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EP, egg white protein by-product; E:S, enzyme: substrate; ELISA, enzyme-linked immunosorbent assay; FRAP, ferric reducing antioxidant power; w/w, weight/weight; HHL, hippuryl-His-Leu; GRAS, generally recognized as safe; IL, interleukin; MALDI-TOF/MS, matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry; LP, lipopolysaccharide; RC, regenerated cellulose; RP-HPLC, reversed-phase high-performance liquid chromatography; eq, equivalent; RPMI-1640 medium, RPMI 1640 medium was developed at Roswell Park Memorial Institute; TFA, trifluoroacetic acid; WPC, whey protein concentrate; YM, yeast-maltose

INTRODUCTION

Proteases are one of the most important groups of enzymes produced on an industrial scale, constituting 60% of the global market of hydrolytic enzymes (Dhurway *et al.*, 2011; Rohrbach *et al.*, 1981; Ray, 2012). They are widely used in the production of foods, nutraceuti-

cals, pharmaceuticals, cosmetics, detergents, as well as in the leather and textile industries, and in waste processing (Bagewadi *et al.*, 2011, Vasantha *et al.*, 2012; Vermelho *et al.*, 2012; Sathyavathan *et al.*, 2013). The use of proteases in industrial processes depends on their substrate specificity, preference for the position of the hydrolyzed peptide bond, the types of substances inhibiting the activity of the enzyme, as well as stability across a wide range of temperature and pH. One of the most important factors is their cost, dependent on the availability of sources and the procedure of isolation (Srinu Babu *et al.*, 2007).

In the food industry, where they play a particularly important role, proteases must be approved for consumption and – in the case of microbial enzymes – cannot be derived from pathogenic microorganisms. Other important safety issues include their toxicological properties, e.g. the absence of impurities such as mycotoxins and antibiotics (Schaafsma, 2009).

Microorganisms serve as the preferred source of proteases due to their rapid growth, the limited space required for their cultivation, and the possibility of easy genetic manipulation to generate new enzymes with altered properties that suit various applications. Many microorganisms secrete proteases into their external environment in order to degrade proteins. Products of this process are then used as nitrogen sources for cell growth. Extracellular enzymes are attractive for isolation procedures, especially on an industrial scale, because such processes do not require complicated technological operations to obtain the enzyme product (Carr, 2002).

Y. lipolytica yeasts possess a high ability to synthesize extracellular proteolytic enzymes. As non-pathogenic organisms, they are generally recognized as safe (GRAS) (Nel *et al.*, 2001), and are present in many food products, mainly dairy products such as cheese, yogurt, and kefir, as well as in sausages, shrimp salads, and soy sauces (Guerzoni *et al.*, 1009). *Y. lipolytica* species belong to a group of non-conventional yeasts with unique physiological features including the ability to assimilate organic compounds. *Y. lipolytica* can exploit glucose, alcohols, acetic acid, and hydrophobic substrates such as fatty acids or alkanes (Dominguez *et al.*, 2000). Depending on cultivation conditions and composition of the substrate, *Y. lipolytica* yeasts reveal different potentials to secrete extracellular proteases. The main factor regulating the expression of a specific enzyme is the pH of the external environment. During growth at a pH above 7.5, serine protease (alkaline extracellular protease - AEP) is expressed (Glover *et al.*, 1997). This enzyme belongs to

the family of subtilisins and has a molecular weight of approximately 30.559 kDa; its sequence is comprised of 297 amino acids. The active site consists of the Asp200, His231, and Ser397 residues, while the Asn292 and Asn330 residues are potential sites of N-glycosylation. This enzyme displays a caseinolytic activity under optimum conditions of pH 9.0 and temperature of 40°C. It also demonstrates an esterase activity towards synthetic substrates containing arginine, lysine, or tyrosine, which it degrades optimally at pH 8.0. The enzyme is stable in a 5.0 to 9.0 pH range, and in the presence of calcium ions (Ogrydziak *et al.*, 2012; 1998).

Recently, proteolytic enzymes have found a wide range of other biotechnological applications, such as bioactive peptide generation, as the result of a precursor protein hydrolysis. The particular researchers' interest in biopeptide production is related to their high therapeutic potential resulting from a broad spectrum of *in vivo* activities, including antioxidant, antibacterial, antihypertensive, immunomodulatory, as well as opiate functions (Zambrowicz *et al.*, 2015b).

Diseases, such as cardiovascular diseases (including hypertension), tumor, obesity, type 2-diabetes or chronic inflammation represent significant health problems worldwide. The role of reactive oxygen species (ROS) in pathogenesis of these diseases is commonly known. The aforementioned dependencies between their etiologies indicate the special importance of multifunctional peptides: their combined antioxidant, ACE-inhibitory and cytokine inducing activity would make a useful peptide preparation for the control and prevention of the so-called civilization diseases (Zambrowicz *et al.*, 2015b; Booth *et al.*, 2012).

The properties of the peptides depend on the nature of the precursor protein, specificity of the protease used and the hydrolysis conditions (the enzyme / substrate ratio, temperature, pH, time). Therefore, industrial demand for proteases with appropriate specificity and stability continues to motivate the search for new sources.

The work presented here reports on the potential application of a non-commercial proteolytic enzyme from the *Y. lipolytica* JIII1c strain to produce biologically active peptides from a hen egg-white protein by-product following lysozyme and cystatin isolation.

MATERIALS AND METHODS

Microorganism. *Y. lipolytica* JIII1c used in this study originated from the yeast culture collection of the Department of Biotechnology and Food Microbiology, Wrocław University of Environmental and Life Sciences in Poland.

Enzyme preparation. Preparation of the serine protease of *Y. lipolytica* was obtained from a submerged culture of yeast in a mineral-organic medium at pH 7.5. Biosynthesis of the enzymes was carried out at 28°C in 300 mL volumetric flasks containing 50 mL of the production medium composed of (g/L): yeast extract (1.7), isoelectric casein (4.0), fatty acids – a by-product of rapeseed oil refining (10.0), KH₂PO₄ (0.5), MgSO₄×7 H₂O (0.25), NH₄Cl (1.0). Inoculum cultures were carried out for 24 hours at 28°C in YM medium, containing (g/L): yeast extract (3.0), maltose extract (3.0), bacto-peptone (5.0), glucose (10.0), and agar (20.0). All of the cultures were cultivated for 24 h. After cultivation, the biomass was centrifuged at 5000×g for 15 minutes at 4°C. The resulting supernatant was concentrated and purified at 4°C using a set of Amicon Bioseparations filtra-

tion membranes of regenerated cellulose (RC) (Millipore) with a nominal cut-off of 10 kDa.

Determination of protein content. Protein content was determined by the method described by Lowry *et al.* (1951).

Determination of proteolytic activity. Enzymatic activity was measured in a reaction with 1% casein (BDH, Ltd. England) and ovalbumin (Sigma) as substrates, at pH 7.5, according to the procedure described previously (Pokora *et al.*, 2010).

EP – egg white protein preparation. EP was obtained in our laboratory as a by-product of lysozyme and cystatin isolation, according to the method of Sokolowska *et al.* (2007).

Enzymatic hydrolysis. EP hydrolysis was carried out according to Zambrowicz *et al.* (2015a), with the protease from *Y. lipolytica* at an E:S ratio of 1:30 (w: w), for 24 hours.

Degree of hydrolysis. The degree of hydrolysis (DH) (%) was measured by determination of a trichloroacetic acid soluble protein fraction (Silvestre, 1996).

Reversed-phase high-performance liquid chromatography (RP-HPLC). Peptide separation was performed by RP-HPLC with an Agilent 1100 Series system using a Zorbax SemiPrep XDB-C₁₈ (5 µm; 9.4×250 mm) and Zorbax XDB-C₁₈ Agilent (50×1.8 mm) columns, at T=30°C. The mobile phase A was 0.1% TFA (Fluka) in water, and the mobile phase B was 0.1% TFA in acetonitrile (LabScan). Other operation conditions were varied; hydrolysate: flow rate 2 mL/min, gradient: from the fifth minute of analysis 1% B/min; fraction 3: flow rate: 1 mL/min, gradient: from the third minute of analysis 4% B/min. The eluents were detected at 230 nm.

DPPH free radical scavenging activity. The antioxidant activities of the obtained hydrolysate and peptide fractions were determined by the radical scavenging effect on stable 1,1-diphenyl-2-picrylhydrazyl (DPPH (Sigma, D21140-0)) by the method described by Yen and Chen (1995) and modified by Zambrowicz *et al.* (2015 a).

Ferric Reducing Antioxidant Power (FRAP). The antioxidant capacity of the hydrolysate/peptide fractions was determined by the FRAP method according to Benzie and Strain (1996). The concentration of Fe (II) was determined using a standard curve for known Fe (II) concentrations (0–500 µg/ml FeSO₄). The results obtained are shown as µg Fe²⁺/mg protein.

Fe (II) ion chelation activity. The ability to chelate the Fe (II) ions by the hydrolysate/peptide fractions was evaluated by the method of Xu *et al.* (2007). Known concentrations of FeCl₂ (0–20 µg) were used to make a standard curve. The ability to chelate iron ions was expressed as µg Fe²⁺/mg protein.

Determination of ACE-inhibitory activity. The ACE inhibitory activity of the hydrolysate and peptide fractions was measured spectrophotometrically according to Miquel *et al.* (2004). A hydrolysate solution (40 µL) mixed with a Hippuryl-His-Leu (HHL) (Sigma, H4884) solution was pre-incubated at 37°C for 5 min, and the reaction was initiated by adding 20 µL (2 mU) of the ACE solution (EC 3.4.15.1; from rabbit lung, Sigma, A6778) and incubated at the same temperature. After 30 min, the enzymatic reaction was halted by the addition of 150 µL of 1 M HCl. The liberated hippuric acid was measured at λ = 228 nm. The inhibition activity was calculated using the following equation:

$$\text{Inhibition activity (\%)} = [(A-B)/A] \times 100\%$$

where A is the reaction blank where the mixture contained the same volume of the buffer solution instead

of the ACE inhibitor sample; B is the reaction in the presence of both ACE and its inhibitor. The IC_{50} value was estimated from a dose response curve of an inhibitor versus the ACE activity.

Cytokine induction in human whole blood cell cultures. Blood samples obtained from healthy donors were collected into sterile syringes containing 10 U/mL of heparin. Within 2 hours of collection, the blood was diluted 10-fold with RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, and 3% L-glutamine. One ml portions of the blood cell suspensions were distributed into 48-well tissue culture plates. The examined inductor (100 μ g) was added to 1 mL RPMI medium. Lipopolysaccharide from *E. coli* (LP; 4 μ g/mL) was used as a positive control. The untreated blood samples were used as a negative control to measure the spontaneous cytokine release. Blood samples were incubated for 22 hours at 37°C in 5% CO₂. After incubation, the supernatants were collected and the levels of IL-6 and IL-10 cytokines were determined by ELISA, using commercially available BD OptEIA™ sets according to the procedure recommended by the manufacturer (Pharmingen, USA).

Molecular weight and sequence of peptides. The molecular weights and sequences of peptide fractions obtained by RP-HPLC were performed using MALDI-ToF analysis UltrafleX-treme apparatus (Bruker Daltonics, Germany) in a positive ion mode. All of the samples were dissolved in 1 ml 60% acetonitrile, centrifuged, evaporated and then dissolved again in 60 μ L of 60% acetonitrile. 40 μ L of this solution was evaporated and then dissolved in 10 μ L of 50% (v/v) acetonitrile. 0.5 μ L of peptide solution was mixed with 0.5 μ L of alpha-cyano-4-hydroxycinnamic acid, pipetted onto a steel plate and evaporated to dryness. The MS and MS/MS spectra were collected and processed using FlexControl, FlexAnalysis, BioTools as well as ProteinScape software (Bruker Daltonics, Germany), while protein sequences were identified using a MASCOT server (MS) (Matrix Science, USA) and the UniProtKB/SwissProt protein sequences database (version 25.10.2012, European Bioinformatics Institute, United Kingdom). Taxonomic search was restricted to *Gallus gallus*, taking into account the possible modifications of polypeptide chains resulting from the oxidation of methionine. No restrictions concerning peptidase specificity during the peptide processing was declared.

Statistical analysis. Experimental hydrolysis was carried out in two independent batches. All analyses of the hydrolysates, as well as the peptide fractions, were done in triplicate for each batch. The data obtained were subjected to multi-factor variance analysis (ANOVA), followed by a Duncan multiple range test to determine the significant difference between samples at $p < 0.05$ using Statistica v. 9.0 software.

RESULTS AND DISCUSSION

Due to its excellent proteolytic properties as well as an easy and cheap process of production, non-commercial serine protease from *Y. lipolytica* JII1c yeast was used to degrade the denatured EP left as a by-product following the isolation of lysozyme and cystatin, in order to obtain hydrolysates exhibiting biological activity. Native egg white proteins, especially ovalbumin, are known as a good source of biopeptides, and are released by the hydrolysis process conducted with digestive proteases (Miguel *et al.*, 2004; Dávalos *et al.*, 2004; Fujita *et al.*, 2000).

Y. lipolytica serine protease is a non-commercial enzyme preparation obtained in our laboratory from a culture broth of yeast cultivated for 48 h. After culturing and centrifugation, the supernatant was ultrafiltered on a cellulosic membrane (cut-off 10 kDa). As a result of this process, the protease has been purified by 1.4 fold with 85% recovery. Its activity was measured toward protein standards, such as casein and ovalbumin. It appeared that this protease exhibited a 2-fold higher proteolytic activity toward casein (713 U/mg) than toward ovalbumin (350 U/mg).

The degradation process of EP was conducted for 24 hours with *Y. lipolytica* serine protease added to the reaction mixture at a dose of E:S=1:30 (w/w). The obtained hydrolysate of EP was characterized by protein degradation (DH) of 38%.

Due to its wide biochemical predispositions, the possibility of applications of *Y. lipolytica* in the biotechnology and food industries has been intensively tested; for example, as factors accelerating cheese ripening (Rywińska *et al.*, 2013; Gardini *et al.*, 2006; Zinjarde, 2014). Still, there is little information on generating bioactive peptides by hydrolysis of food proteins with this enzyme. Therefore, in the research presented here we tested the biological activities of EP hydrolysates and the isolated peptides. The attractiveness of serine protease obtained using *Y. lipolytica* was demonstrated in the evaluation of the antioxidant and ACE inhibitory (for selected peptide fractions) activities of the EP hydrolysates. A high ability to chelate iron (II) at 688.5 μ g Fe²⁺/mg was demonstrated, with a reducing power, measured as ferric to ferrous ion reduction ability, of 35.3 μ g Fe²⁺/mg. A noticeable free radical scavenging activity (0.21 μ M Trolox_{eq}/mg) was also observed (Table 2).

Strong proteolytic properties toward milk proteins, including casein and whey proteins, have been demonstrated in a few earlier publications. Babij *et al.*, (2013), who reported that α -lactalbumin, WPC-50 preparation and β -lactoglobulin can be hydrolysed by *Y. lipolytica* protease with different efficacy to a DH of 18%, 23% and 39%, respectively. They also confirmed in a further work the high ability of this protease to release peptides with antioxidant activities. Degradation of WPC-80 preparations led to hydrolysates with significant DPPH free radical scavenging activity (0.81 μ M Trolox_{eq}/mg) (Babij *et al.*, 2014). However, among the milk proteins, casein was the most susceptible to degradation with this protease and after 24 hours hydrolysis DH reached a level of 66%. The obtained products displayed almost the same level of ferric reducing power (39.0 μ g Fe²⁺/mg), as in the present study. Interestingly, casein was a better source of peptides than egg yolk protein byproduct, having chelating (1559.0 μ g Fe²⁺/mg) and free radical scavenging activities (0.86 μ M Trolox_{eq}/mg) released by the participation of serine protease from *Y. lipolytica* (Szoltyśik *et al.*,

Table 1. Induction of IL-6 and IL-10 by human whole blood cells in response to EP.

1 ml portions of human whole blood cells were incubated at 37°C for 22 hours in the presence of the analyzed samples (1-100 μ g/ml). Lipopolysaccharide (LP) (4 μ g) was used as a positive control where cells were incubated in the absence of inducers. Supernatants were harvested and analyzed for IL-6 levels by ELISA, as described in Materials and Methods.

Sample	IL-6 [pg/ml]	IL-10 [pg/ml]
Control	0	0
LP	<1000±50	361±110
EP hydrolyzate	540±27	342±262

Table 2. The antioxidant activity of the fractions obtained by RP-HPLC separation of the EP hydrolysate obtained with the use of *Yarrowia lipolytica* serine protease.

The data shown in the table are means (mean \pm S.D., n=3). The mean values marked in lower case letters in the same column are significantly different at $p \leq 0.05$.

Fraction number	DPPH [μ M Trolox _{eq} /mg]	FRAP [μ g Fe ³⁺ /mg]	Fe (II) chelating [μ g Fe ²⁺ /mg]
hydrolysate	0.21	35.3	688.5
1	0.04 ^c	8.05 ^h	44.88 ^c
2	0.02 ^a	4.21 ^c	25.43 ^a
3	0.16 ^f	12.22 ⁱ	111.99 ^f
4	0.04 ^c	12.48 ^j	77.70 ^e
5	0.06 ^e	13.62 ^j	74.84 ^e
6	0.04 ^c	6.31 ^{ef}	55.16 ^d
7	0.03 ^b	5.65 ^{de}	45.72 ^c
8	0.02 ^a	2.92 ^b	36.06 ^b
9	0.02 ^a	3.41 ^b	21.73 ^a
10	0.02 ^a	2.09 ^a	24.92 ^a
11	0.02 ^a	5.60 ^d	46.94 ^c
12	0.04 ^c	6.52 ^f	56.80 ^d
13	0.03 ^b	5.13 ^d	206.96 ^h
14	0.05 ^d	7.18 ^g	120.52 ^g
15	0.04 ^c	7.47 ^{gh}	80.82 ^e

2012). However, DH – which was 1.7 fold higher for the casein hydrolysate – may be a factor influencing the level of antioxidant activity, as this depends not only on the substrate and the degree of hydrolysis, but also on the type of the enzyme applied in the process.

On the other hand, serine protease from *Y. lipolytica* was characterized by a lower ability to hydrolyze the EP preparation than other microbial proteases, such as Neutrased from *Bacillus amyloliquefaciens* and an *Aspergillus melles* protease. These proteases, applied at a dose of 10 U per mg of EP, resulted in a DH after 24 h reaction of nearly 75% and 60%, respectively. The degradation of EP with the use of Neutrased and protease from *A. melles* led to hydrolysates with positive ferric reducing and ferric chelating properties, 21.0 and 5.9 μ g Fe²⁺/mg, and 397.0 and 278.1 μ g Fe²⁺/mg, respectively (Zambrowicz *et al.*, 2013). The results indicate that serine protease from *Y. lipolytica* is characterized by excellent properties for releasing antioxidant peptides from EP in comparison to the commercially available microbial enzymes cited above.

Cytokine induction was determined under *ex vivo* stimulation of human whole blood cell cultures with LP and the examined EP hydrolysate. This method imitates the natural microenvironment of immunocompetent cells and preserves the intercellular communications between the different blood cell populations. It was observed that EP hydrolysate, at a dose of 100 μ g/ mL was able to induce the secretion of IL-6 and IL-10 by human whole blood cells (Table 1). It was shown that EP hydrolysate stimulates the blood cells to release the anti-inflammatory IL-10 in amounts comparable to LP used as a positive control. Also, the level of pro-inflammatory IL-6 produced in response to EP hydrolysate was significantly higher compared to the untreated cells, although almost 50% lower than that observed for LP.

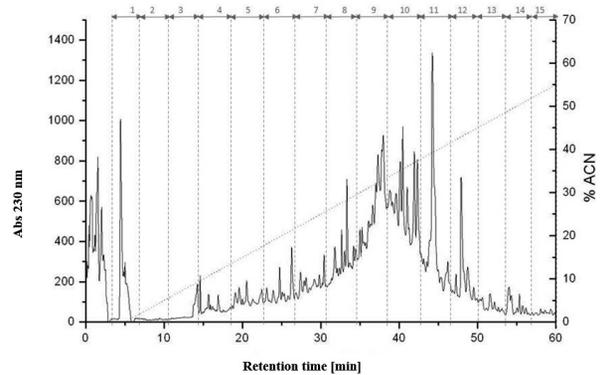


Figure 1. Fractionation of the EP hydrolysate by RP-HPLC.

Separation was performed on a Zorbax SemiPrep XDBC18 (5 μ m, 9.4 \times 250 mm) column equilibrated with 0.1% TFA in water (phase A). Separation conditions: time – 65 min., T=30°C, flow 2 mL/min, gradient 0–100% B (0.1% TFA in acetonitrile).

In summary, the results obtained here show that EP hydrolysate is a good stimulator of cytokine production in human whole blood cell cultures. Interleukin 6 and Interleukin 10 are produced by Th2 cells and play a crucial role in the regulation of the humoral immune response. The results presented here suggest the possibility of using EP hydrolysate to induce Th2-type cytokines and to regulate the inflammatory reaction.

The isolation and identification of the peptides that determine specific biological activities require the hydrolysate to be fractionated. It is well known that separation of complex mixtures, such as products of enzymatic hydrolysis, is difficult and requires the use of a combination of techniques using the different properties of the peptides (Kamysz *et al.*, 2004). In our research, a biopeptide fractionation procedure based on ultrafiltration and RP-HPLC was used. Initially, the hydrolysate was separated using an ultrafiltration membrane with a cut-off at 30 kDa (Amicon) to remove the enzymes and non-hydrolyzed EP. Subsequently, the hydrolysate was fractionated by RP-HPLC using a Zorbax SemiPrep XDBC18 column (5 μ m, 9.4 \times 250 mm). This method is regarded as a standard and the most effective of the procedures used in the isolation of proteins and peptides. The main advantage of RP-HPLC is that it allows the separation of even very small fragments of proteins with similar

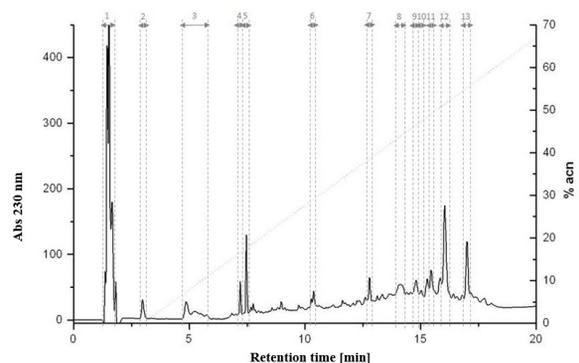


Figure 2. The isolation of peptides from fraction number 3 by RP-HPLC.

The sample was loaded onto a Zorbax XDB-C18 Agilent (50 \times 1.8 mm) column equilibrated with 0.1% TFA in water (phase A). Separation conditions: time – 15 min., T=30°C, flow 1 mL/min, gradient 0–100% B (0.1% TFA in acetonitrile).

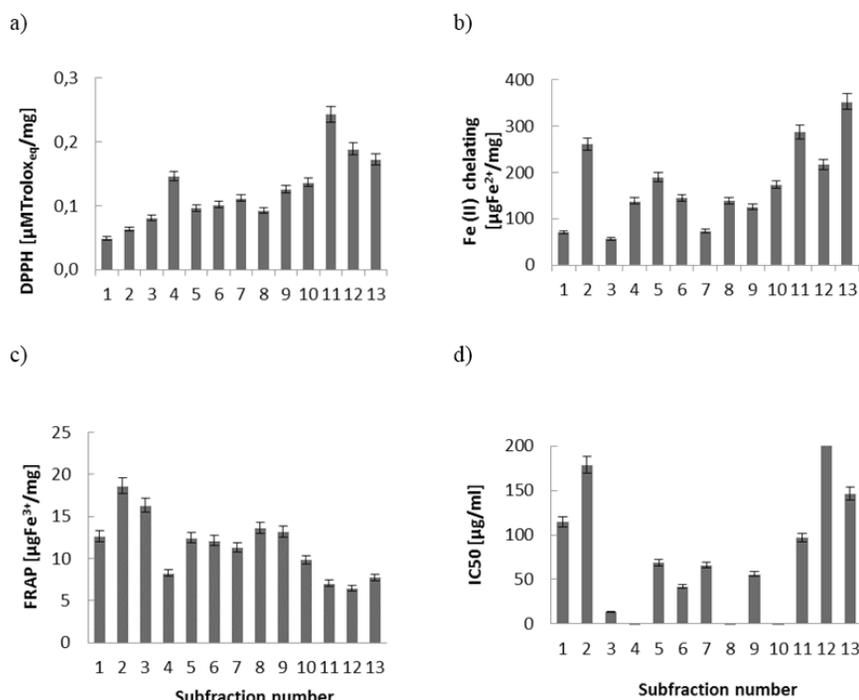


Figure 3. The biological activity of peptide subfractions collected during the chromatography process of fraction number 3. (a) the DPPH free radical scavenging ability; (b) the ability to chelate Fe (II), (c) FRAP; (d) ACE inhibitory activity.

molecular weights and only slightly different amino acid sequences. As a result of RP-HPLC separation, 15 peptide fractions of different hydrophobicity were obtained (Fig. 1). The results show that all of them exhibited antioxidant activity at different levels, but in all cases they were lower than for the initial hydrolysate (Table 2). Similar observations have been described by other researchers, highlighting that in some cases the purification of hydrolysates leads to a loss of their antioxidant activity, which frequently is a result of the entire system of interactions consisting of peptides and free amino acids (Li *et al.*, 2007). However, the highest antioxidant activity, especially a DPPH radical scavenging activity of 0.16 $\mu\text{M Trolox}_{\text{eq}}/\text{mg}$, as well as ferric reducing antioxidant power (12.22 $\mu\text{g Fe}^{2+}/\text{mg}$) and ferrous chelating (111.9 $\mu\text{g Fe}^{2+}/\text{mg}$) power, was revealed by fraction number 3 (Table 2). This fraction was subjected to further purification using RP-HPLC on a Zorbax XDB-C₁₈ column (1.8 μm , 4.6 \times 50 mm) (Fig. 2). As a result of this separation step, 13 peptide subfractions were obtained and then were tested for both, the antioxidant and ACE inhibitory activities (Fig. 3).

Four of the obtained subfractions (4, 11, 12, 13) showed higher or similar ability to scavenge free radicals in relation to fraction number 3, in the range of 0.15 to 0.24 $\mu\text{M Trolox}_{\text{eq}}/\text{mg}$. Evaluation of the reducing power (FRAP) revealed the highest value (from 13.2 to 18.6 $\mu\text{g Fe}^{2+}/\text{mg}$) in the case of subfractions 2, 3, 8 and 9. Ten of thirteen of the obtained subfractions showed a significantly higher ability to chelate iron (II) than fraction no. 3, in the range of 125.5–353.7 $\mu\text{g Fe}^{2+}/\text{mg}$.

Food proteins, especially egg proteins, are a rich source of ACE inhibitory peptides (Majumder & Wu, 2011). The ability to release ACE inhibitory peptides by *Y. lipolytica* serine protease was also analyzed. The obtained subfractions exhibited ACE inhibitory activity at IC₅₀ values in the range from 13.7 to 1229.1 $\mu\text{g}/\text{mL}$. Subfractions number 3 (IC₅₀=13.7 $\mu\text{g}/\text{mL}$) and 6

(IC₅₀=42.1 $\mu\text{g}/\text{mL}$) were the strongest ACE inhibitors. In a previous work, the effects of enzymatic modification of egg white protein by-product (EP) on biological properties were assessed by treatment with Neutrase. The EP hydrolysate, obtained at a technical scale, had an *in vitro* ACE inhibitory effect of IC₅₀=257.4 $\mu\text{g}/\text{mL}$ (Pokora *et al.*, 2013).

Peptide subfractions showing the highest biological activity were identified using MALDI-TOF/MS and the Mascot Search Results database. The representative MALDI-ToF/MS spectra are shown in Fig. 4. These analyses showed that the subfractions were mostly mixtures of at least two peptides with sequences corresponding to the hen egg proteins, containing from 2 to 19 amino acid residues (Table 3).

Subfractions 5, 6 and 13 were homogeneous peptides corresponding to ovalbumin fragments with the sequences: VGS (f 343–354), GV (f 349–350) and YQI-GLFRVASMASEKM (f 212–228), respectively. Analysis of peptide subfractions 11, 12, and 13 showed that they were mixtures of several peptides. The identification of parent ions by MALDI/TOF/MS revealed the presence of three peptides in subfraction 11: VLDRTWP-PAP, TEAMKRALALIDS, and GGVGVPGGLGVPG-VVQPGV, corresponding to the fragments of chicken myosin, chicken vinculin, and chicken elastin, respectively (Fig. 4). In subfraction 12, only fragments of ovalbumin with sequences RFDKLPFGFSDSIEAQ (f 59–72), KMKILELPPFASGTM (f 227–240), and LELPAFASGTM (f 231–240) were identified. Subfraction 2 contained two peptides, DTALGHVI and SSGGAGTTPPA, corresponding to the fragment of the integrator complex subunit 10 and HMX1 homeobox protein, respectively.

The amino acid sequence and composition constitute the main factor determining the bioactivity of the peptides (Danquah & Agyei, 2012). The hydrophobic properties of peptides play a significant role in their antioxidant activity, particularly in the ability to scavenge

free radicals and in their reducing power (FRAP). The presence of such amino acid residues as G, A, V, I, L, P, Y, M, H, or W in the peptide sequence denotes high antioxidant properties (Wang *et al.*, 2009). Hydrophobic amino acids are present in the sequences of peptides identified in subfractions 11, 12 and 13, which can result in their high DPPH radicals scavenging capacity, at 0.24, 0.19, and 0.17 μM Trolox_{eq}/mg, respectively. Moreover,

the presence of indole, phenol, or imidazole groups (W, Y, H) in the side chain, as the donors of hydrogen ions, also enhances the antioxidant activity of the peptides (Ajibola *et al.*, 2011). Guo *et al.* (2009) had reported that strong antioxidant activity is also determined by the N-terminal location of the L, I, V, K, and A residues in the peptide chain. This may explain the activity of several peptide fractions in which peptides with particularly longer chains were identified, e.g. KMKILELPAFASGTM, LELPAFASGTM, and VLDRTWPPAP.

The ability to chelate divalent cations of metal ions is related to the presence in the peptide sequence of branched chain amino acids, such as E, Q, L, R, D, H, and T. The side chains of these amino acids can react with metal ions and thereby inactivate their pro-oxidant action. The presence of such amino acids may explain the highest iron ion chelating activity of subfractions 11 and 13. Amino acid sequence analysis of these subfractions has demonstrated the presence of peptides containing glutamic acid, aspartic acid, glutamine, lysine, and threonine. Elias *et al.* (2008) pointed out the location of branched chain amino acids in the sequence as an important factor in the chelating activity of peptides and proteins. It is particularly advantageous if they are exposed on the surface of the protein or at the ends of the peptide chains. Such a structure is characteristic for the peptides TEAMKRALALID, LELPAFASGTM, and DTALGHVI, identified in subfractions 11, 12, and 2, respectively.

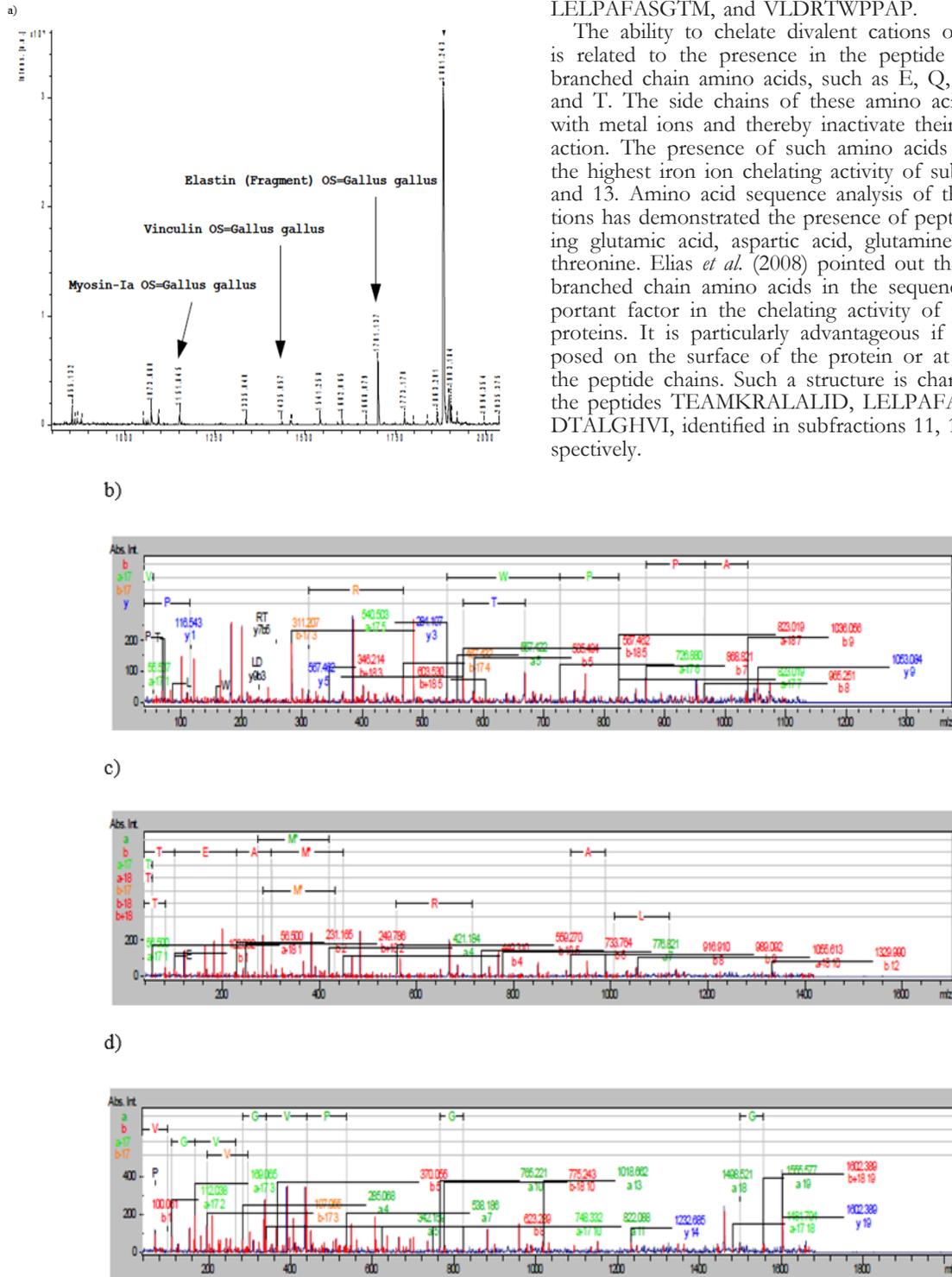


Figure 4. Peptide identification by MALDI-TOF-MS mapping.

(a) representative MALDI-TOF-MS spectra of subfraction 11; (b–d) spectra of selected fragment ions, (b) chicken myosin peptide – VLDRTWPPAP, (c) chicken vinculin peptide – TEAMKRALALIDS, (d) chicken elastin peptide – GGVGPGLGVPVGVQPVG.

Table 3. Identification of peptide fragments in the selected peptide subfractions.

Subfraction number	Peptide sequence MALDI TOF/ MS	Biological activity	Homolog protein/ UniProtKB/SwissProt
2	DTALGHVI [1]; SSGGAGTPPPA [2]	FRAP; Fe (II) chelating	[1] – fragment subunit 10 of the chicken egg complex integration [2] – HMX1 homeobox protein fragment
5	VGS	ACE inhibitor	ovalbumin, (f 343–345)
6	GV	ACE inhibitor	ovalbumin, (f 349–350)
11	VLDRTWPPAP [1]; TEAMKRALALIDS [2]; GGVGVPGGLGVPVVQPGV [3]	DPPH scavenging, FRAP; Fe (II) chelating	[1] – fragment of chicken myosin, [2] – fragment of chicken vinculin [3] – fragment of chicken elastin;
12	RFDKLPFGDSIEAQ [1]; KMKILELPFASGTM [2]; LELPAFASGTM [3]	DPPH scavenging, Fe (II) chelating	[1] – ovalbumin, (f 59–72) [2] – ovalbumin, (f 227–240) [3] – ovalbumin, (f 231–240)
13	YQIGLFRVASMASEKM	DPPH scavenging, Fe (II) chelating	ovalbumin, (f 212–228)

A common feature of several of the identified peptides is the presence of repeating amino acid residues located in adjacent positions in the peptide structure; e.g. the dipeptide and tripeptide sequences of serine, glycine, proline, or valine in the peptides SSGGAGTPPPA and GGVGVPGGLGVPVVQPGV. According to Siow & Gan (2013), such peptide fragments have a higher biological activity than sequences containing such amino acid appearing only once.

Food proteins are a rich source of peptide inhibitors of the angiotensin-converting enzyme (ACE), an enzyme which plays a crucial role in the regulation of blood pressure. In our study, a strong ACE inhibitory effect was exerted by subfractions 3, 6, 7, and 5. Among them, MS analysis of subfractions 5 and 6 gave positive results, indicating that they are homogenous and composed of short chain peptides VGS and GV, respectively. According to other authors, to generate an inhibition effect, ACE reacts with peptides composed of specific amino acid residues, typical for C- and N ends of the ACE inhibitors. Hydrophobic amino acids, especially those with aliphatic chains, such as G, L, I and V, are typical for the N- end of peptides (Iwaniak *et al.* 2014). The presence V and G may explain the high antihypertensive effect of peptides from EP. Furthermore, the most potent ACE inhibitors contain hydrophobic amino acid residues at their C-terminal tripeptide sequence (Karamać *et al.*, 2005). In our study, subfraction 11, composed of the VLDRTWPPAP, TEAMKRALALID, and GGVGVPGGLGVPVVQPGV peptides, exerted a significant ACE-inhibitory activity. The first peptide contains two proline residues while the third contains proline, glycine, and valine at the C-terminal tripeptide sequence, which may be crucial for the ACE-inhibitory activity.

The biopeptides obtained in our study have not been previously described by other authors. However, peptides having some sequence similarity are known. An example would be the sequences of VG and PG with potential ability of ACE inhibition, identified in *in silico* studies with bovine lactoferrin, as well as the VECYGP-NRPQF peptide isolated from a pepsin hydrolysate of algae protein. There are also known peptides with antihypertensive activity, such as SY, NY, SF, and NF, whose characteristic feature is the presence of serine or asparagine at the N-terminus (Kumar *et al.*, 2010).

CONCLUSIONS

Tests were made of the proteolytic properties of the non-conventional serine protease from *Y. lipolytica* toward egg white protein by-product (EP) following the ethanol extraction of lysozyme and cystatin, and its ability to release bioactive peptides. This serine protease had caused an extensive degradation of EP (38%). The obtained hydrolysate exhibited antioxidant and cytokine inducing activity. The purification and characterization of the EP-derived peptides by ultrafiltration, RP-HPLC, and MALDI ToF mass spectrometry led to detection of the following peptide sequences DTALGHVI, SSGGAGTPPPA, VGS, GV, VLDRTWPPAP, TEAMKRALALIDS, GGVGVPGGLGVPVVQPGV, RFDKLPFGDSIEAQ, KMKILELPFASGTM, LELPAFASGTM, and YQIGLFRVASMASEKM. The obtained peptide fractions exerted an ACE-inhibitory activity and an antioxidant activity reflected as the DPPH free radical scavenging ability, ability to chelate Fe (II), and a ferric reducing activity (FRAP). A new application of the serine protease from *Y. lipolytica* yeast in the production of bioactive peptides was shown.

Conflict of interest

All of the authors who took part in this study declare that they have nothing to disclose regarding competing interests or funding from industry with respect to this manuscript.

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