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# ANTIOXIDANT ACTIVITY OF CHICKEN EMBRYO TISSUES POWDER OBTAINED BY DIFFERENT METHODS OF HYDROLYSIS

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## Abstract

Despite the unanimous opinion on the high biological activity of various extracts of chicken embryo tissues, the information on the active principles are contradictory. However, most researchers agree with the opinion on the importance of the antioxidant activity of amino acids and low molecular peptides. The purpose of this study was to assess the effect of various methods of chicken embryonic tissues preparation (acidic, enzymatic and mixed) on their antioxidant activity.

Certified fertilized chicken eggs were produced by white Leggorn breeds of Kumskaya poultry (Russia) set. Cultivation of embryos up to 10 days of age was carried out under laboratory conditions in an incubator ILB-0.5 (Russia) with automatic regulation of incubation parameters. Defatted powder of chicken embryo tissues were subjected to acidic, enzymatic and several types of mixed hydrolysis to verify their antioxidant activities, evaluated by the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging, lipid peroxidation inhibition, and an electrochemical method. The degree of hydrolysis were determined by the amine nitrogen/total nitrogen ratio in the final hydrolysate.

In all the hydrolysates obtained there is a wide list of amino acids, however the poorest in composition were acid, mixed pepsin and mixed tripsin hydrolysates. The chemical data of the mixed hydrolysates obtained using porcine pepsin and pancreatin and pancreatin showed that the most abundant products are amino acids and small peptides. In addition, all mixed hydrolysates obtained with the use of pancreatin showed high antioxidant activity as well as a high level of inhibition of lipid oxidation with respect to trolox.

However, taking into account complexity of hydrolysis technology and raw materials cost, combined method of chicken embryonic tissues hydrolysis with application of HCl and pancreatin (MHpancreatin2) is recommended for industrial implementation. The hydrolysate obtained by this method contains peptides, has antioxidant activity, angiotensin-converting-enzyme (ACE) inhibitor activity, activity of a dipeptidyl peptidase IV inhibitor, as well as antibacterial activity. These properties can be useful to employ this hydrolysate as food additives, pharmaceutical agents and as food components to prevent oxidative reactions.

***Key words**: Acidic, Antioxidant activity, Chicken embryo tissues, Enzymatic and mixed hydrolysis, Physico-chemical property.*

## 1. Introduction

Searching for natural biological substances as sources of raw materials for peptide-containing drugs is today a promising direction of pharmacy.

In the literature, placental extracts are the most often refers. They were used since many years in many countries for healing wounds, treatment of chronic inflammatory diseases and for cosmetic purposes (Tonello *et al.,* [34]). In addition to biologically active peptides, placental extracts contain enzymes, nucleic acids, vitamins, amino acids, steroids, fatty acids and minerals. Placental extracts induce immunotropic, antioxidant and anti-inflammatory reaction (Biswas *et al.*, [4]; Park *et al.*, [26]; Togashi *et al.,* [33]; Yeom *et al.*, [37]).

However, the placenta is not always available as raw material, and depending on the physiological features of its development, it can necrotize or contain toxic components.

Therefore, now the task of searching for non-placental tissues as material containing biologically active components and proteins is considered topical. As source of raw materials, chicken embryos, which are chemically not inferior to human placental tissues and are traditionally used as natural food additives in China and South-East Asia, can be considered a valuable substitute (Gao *et al.*, [8]; Liu, [18]; Li Xi *et al.*, [16]).

The study of extracts of chicken embryonic tissues revealed that they possess regenerative stimulating, hepatoprotective, and immunomodulating effect, as well as pronounced anti-inflammatory effect (Areshidze *et al*., [1]; Wang *et al.*, [36]).

However, it was found that chicken extracts have significant biological activity, including to: relieve stress and fatigue, reduce anxiety, stimulate metabolism, particularly in the period of postpartum lactation, normalize the state in hyperglycemia and hypertension, increase immunity, and so on. These activities can be associated to their active components, such as polypeptides, peptides (including such as carnosine and anserine), trace elements, as well as amino acids. The basic mechanisms responsible for the bioactivity of chicken extracts are mainly associated to their antioxidant action and to the effect on nervous regulation (Areshidze et al., [3]; Kim *et al.*, [12]; Kojima et al., [13]; Li *et al.*, [17]; Meng *et al.*, [22]; Sakanaka *et al.,* [29]; Sakanaka and Tachibana, [31]).

It was shown that the antioxidant activity of the protein hydrolysate of chicken embryonic tissues increases with a degree of hydrolysis a greater proportion of smaller peptides (Chay Pak Ting *et al.*, [5]; Lee *et al.*, [15]; Lu and Baker, [19]; Mendis *et al.*, [20]; Park *et al.*, [26]; Sakanaka *et al.*, [30]).

In addition, Chay Pak Ting *et al.*, [5], found that histidine (His), lysine (Lys), methionine (Met), leucine (Leu), phenylalanine (Phe) and arginine (Arg) were the main amino acids that reflect the antioxidant activity of various extracts of the chicken egg.

The above facts indicate that the most interesting therapeutic properties of the extracts of chicken embryo tissues is due to the content of low-molecular compounds: peptides (containing Leu in particular at their N-terminal position and below 5 kDa), and amino acids (His, Lys, Met, Leu, Phe and Arg). These results also suggest that the biological activity of the protein hydrolysate may be related to the synergistic effect of a particular amino acid composition, sequence and molecular weight of the peptides (Chay Pak Ting *et al.*, [5]).

In the literature, (Areshidze *et al.*, [2]; Li Xi *et al.*, [16]; Li *et al.*, [17]; Wang *et al.*, [36]) different preparation of enzymatic hydrolysates or homogenates purified in various ways as extracts of chicken embryo tissues and chicken protein, are presented. However, these ways of obtaining extracts require a lot of time, are expensive, and also require considerable effort to purify them, because of the presence of many high-molecular compounds. To solve this problem, a variety of methods of hydrolysis are proposed together with the use of membrane ultrafiltration, to remove high molecular non-hydrolysed compounds and proteolytic enzymes (Chay Pak Ting *et al.*, [5]; Turgeon and Gauthier, [35]).

Thus, despite the unanimous opinion on the high biological activity of various extracts of chicken embryo tissues, the information on the active principles are contradictory, which is probably due to the specificity of obtaining certain extracts. However, most researchers agree with the opinion on the importance of the antioxidant activity of protein components, such as amino acids and low molecular peptides.

The purpose of this study was to assess the effect of various methods of chicken embryonic tissues preparation (acidic, enzymatic and mixed) on their antioxidant activity. This will allow the use of these functional ingredients in food manufacturing taking in account their quality and cost.

## 2. Materials and Methods

### 2.1 Materials

Certified fertilized chicken eggs were produced by white Leggorn breeds of Kumskaya poultry (Russia) set. Cultivation of embryos up to 10 days of age was carried out under laboratory conditions in an incubator ILB-0.5 (Russia) with automatic regulation of incubation parameters. During the incubation, the viability and level of embryos development was monitored using the PKYA-10 ovoscope (Russia). In accordance with the patent of the Russian Federation No 2560845 (Timchenko *et. al.*, [32]), stimulation of embryos devel-

opment was carried out using the AL-01 Semicon (Russia) medical semiconductor laser device. On the 10th day of incubation, eggs with developed embryos were placed for 7 days in a refrigerator at 2 - 6 $^0$C. The embryonic and extraembryonic tissues were then separated from the shell and ground using a Sterilmixer 12 knife homogenizer (PBI, Italy). The obtained substance was dried in a laboratory lyophilic dryer LS-500 (Russia) and stored at 20 $^0$C until use.

Chemicals were obtained from the following sources: porcine pepsin (activity 600 - 1800 U/mg, Sigma-Aldrich), pancreatin (activity: amylase 22500 FIP E/g, lipase 22500 FIP E/g, protease 1050 FIP E/g, AppliChem), trypsin (activity 10000 U/mg, Sigma-Aldrich), pepton (total nitrogen 14.22%, Diaem, Russia), petroleum ether 40 - 70 98%, concentrated hydrochloric acid 35% GOST 3118-77, antioxidant assay kit: buffer (Tris-HCl buffer (20 mmol/L, pH 7.4) and 0.95 mmol/L trolox solution), reagent (chromogen containing ABTS- radical ($ABTS^+$)), standards (trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)) (Institute of bioorganic chemistry, National academy of sciences of Belarus, republic of Belarus), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Sigma-Aldrich), oleic acid (Sigma-Aldrich), gallic acid (Sigma-Aldrich), picric acid (Sigma-Aldrich), D-(+)-Glucose monohydrate (AppliChem), standards for chemical research were purchased from Diaem (Russia). Ultrapure water (type I by ASTM) (Milli-Q, Millipore, USA), deionized water, ethyl alcohol 96% (Diaem, Russia), formic acid, ~ 98% (Sigma-Aldrich), acetonitrile (Sigma-Aldrich), α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich), trifluoroacetic acid, > 99% (Sigma-Aldrich), MBT test standard for internal mass spectrometer calibration (Bruker Daltonics).

## 2.2 Methods

2.2.1 Preparation of hydrolysates

Several variants of hydrolysates were obtained during the study: acidic, enzymatic and five mixed. The raw material was a sublimated embryonic-egg mass from which the lipid fraction was removed by 5-fold extraction with petroleum ether. It was stirred at 500 rpm on the magnetic stirrer (Heildoph, Germany), followed by drying of the defatted residue at 37 $^0$C in an ES 20/60 thermoshaker (Biosan, Latvia). The resulting sublimate was carefully ground to a powdery state.

*2.2.1.1 Preparation of acidic* hydrolysate

To obtain acid hydrolysate (AH) 500 mL of distilled water (DW) was added to 20 g of protein-containing powder, mixed and placed in an ES 20/60 shaker-thermostat for 30 minutes at 50 $^0$C. Then, 35% HCl was added to the solution to a final concentration of 0.5% and held at 50 $^0$C for 60 minutes with shaking. The resulting mass was then autoclaved at 125 $^0$C for 60 minutes in a SPVA-75-1NN steam sterilizer (Trans-signal, Russia). The resulting hydrolysate was neutralized with 1M NaOH to pH 6.8-7.2 (pH meter S400-B (Mettler Toledo, Spain)) and centrifuged (SL40R centrifuge (Thermo fisher scientific, USA)) at 4700 rpm for 120 minutes at a temperature of 2 - 4 $^0$C. The liquid obtained after centrifugation was subsequently filtered to remove non-hydrolysed proteins using a Vivaflow 50 filtration system (Sartorius, France) with 0.2 μm and 30 kDa, 10 kDa MWCO polyether sulfone membranes. The resulting filtrate was autoclaved at 120 $^0$C for 10 minutes.

*2.2.1.2 Preparation of enzymatic hydrolysate*

The enzymatic hydrolysate (EH) was prepared per the recommendations of Li Xi *et al.*, [16].

EH was prepared using porcine pepsin and pancreatin. Five hundred ml of DW were mixed with 20 g of protein-containing powder were mixed and putted into an ES 20/60 shaker-thermostat for 30 minutes at 50 $^0$C. Then, 35% HCl was added to the solution to a final concentration of 0.5%. The mixture was incubated with 0.1% porcine pepsin in an ES 20/60 shaker-thermostat for 120 min at 37 $^0$C. The sample was then neutralized with 1 M NaOH to pH 7.0-7.3 (S400-B pH meter) and pancreatin 2 mg/mL was added and incubated in a ES 20/60 shaker thermostat for 120 minutes at 37 $^0$C. Hydrolysis was stopped by boiling for 10 minutes. The resulting hydrolysate was centrifuged (SL40R cooled centrifuge) at 4700 rpm for 120 minutes at 2 - 4 $^0$C. The liquid obtained after centrifugation was subsequently filtered to remove enzymes and non-hydrolysed proteins using a Vivaflow 50 filtration system with 0.2 μm and 30 kDa, 10 kDa MWCO polyether sulfone membranes. The resulting filtrate was autoclaved at 120 °C for 10 minutes.

*2.2.1.3 Preparation of mixed hydrolysates*

Mixed hydrolysates were obtained by sequential application of acidic and enzymatic hydrolysis.

- *Mixed hydrolysate* № 1 (MHpp). Five hundred mL of DW were mixed with 20 g of a protein-containing powder, and putted into a ES 20/60 shaker-thermostat for 30 minutes at 50 $^0$C. Then, 35% HCl was added to the solution to a concentration of 0.5% and held at 50 $^0$C for 60 minutes with shaking 100 rpm in an ES 20/60 shaker-thermostat. The resulting mass was then autoclaved at 125 $^0$C for 60 minutes in the SPVA-75-1NN steam sterilizer. Porcine pepsin (0.1 %) was added to a cooled substance at a concentration of 0.1% and the mixture was incubated in an ES 20/60 shaker-thermostat for 120 minutes at 37 $^0$C.

The sample was then neutralized with 1 M NaOH to pH 7.0-7.3 (S400-B pH meter) and pancreatin 2 mg/mL was added and incubated in a ES 20/60 shaker thermostat for 120 minutes at 37 °C. Hydrolysis was stopped by boiling for 10 minutes. The resulting hydrolysate was centrifuged (SL40R cooled centrifuge) at 4700 rpm for 120 minutes at 2 - 4 °C. The liquid obtained after centrifugation was subsequently filtered to remove enzymes and non-hydrolysed proteins using a Vivaflow 50 filtration system with 0.2 µm and 30 kDa, 10 kDa MWCO polyether sulfone membranes. The resulting filtrate was autoclaved at 120 °C for 10 minutes.

- *Mixed hydrolysate № 2* (MHpepsin). This was prepared as mixed hydrolysate № 1 except that porcine pepsin was added to a cooled substance at a concentration of 0.1% and the mixture was incubated in an ES 20/60 shaker-thermostat for 120 min at 37 °C. The sample was then neutralized with 1M NaOH to pH 7 - 7.3 (S400-B pH meter). Hydrolysis followed as for mixed hydrolysate № 1.

- *Mixed hydrolysate № 3* (MHpancreatin2). This was prepared as mixed hydrolysate № 1 except 2 mg/mL of pancreatin was added to the steam sterilized mass after cooling. Hydrolysis followed as for mixed hydrolysate № 1.

- *Mixed hydrolysate № 4* (MHpancreatin4). This was prepared as mixed hydrolysate № 1 except 4 mg/mL of pancreatin was added to the steam sterilized mass after cooling. Hydrolysis followed as for mixed hydrolysate № 1.

- *Mixed hydrolysate № 5* (MHt). This was prepared as mixed hydrolysate N. 1 except that 25 µg/mL of trypsin was added to the steam sterilized mass after cooling. Hydrolysis followed as for mixed hydrolysate № 1.

The general scheme for obtaining hydrolysates of chicken embryonic tissues is presented at Figure 1.

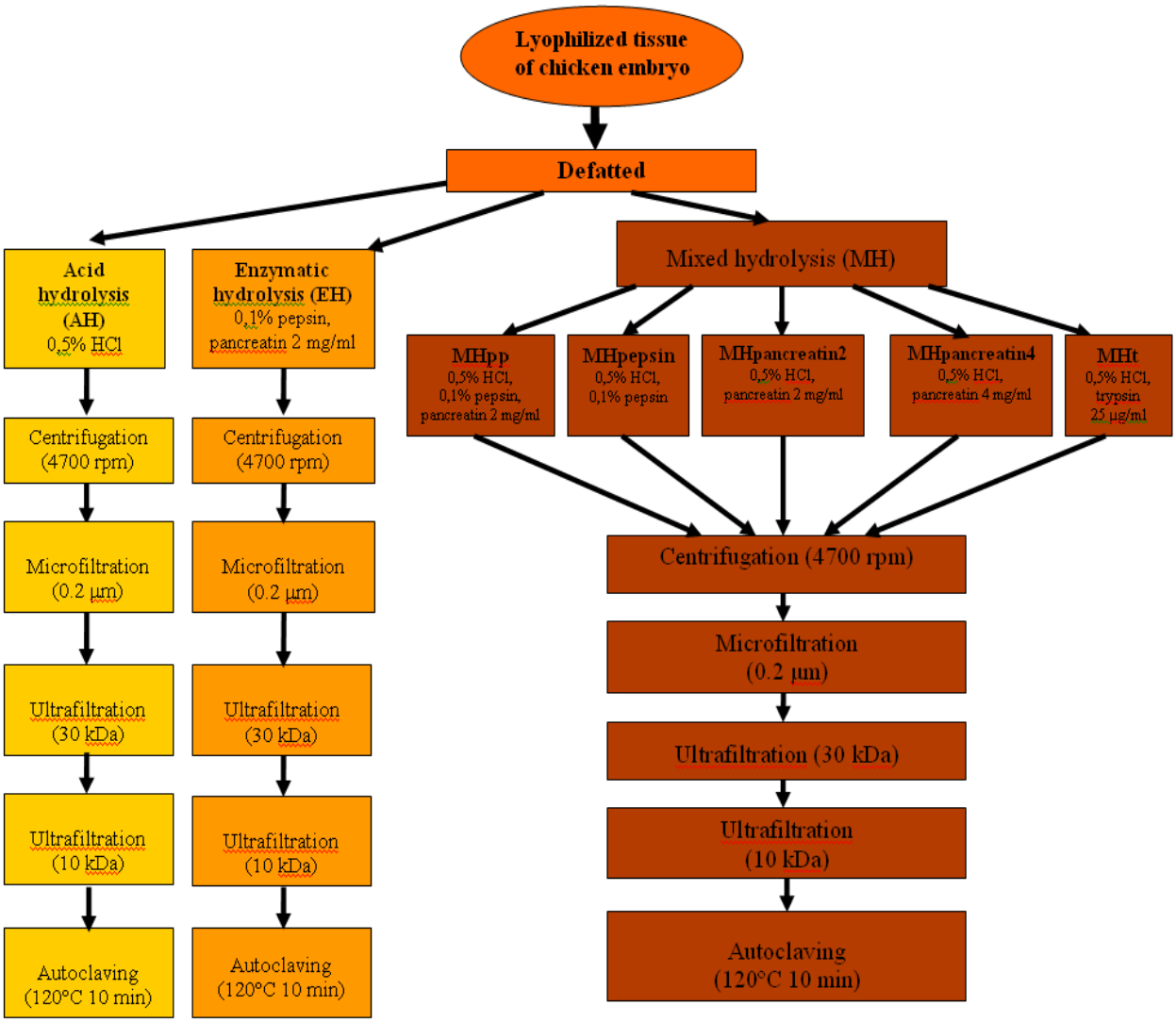


**Figure 1. Schematic representation of the process used for production of hydrolysates**

*2.2.1.4 Physico-chemical characterization of the hydrolysates*

*- Hydrolysis degree*

The degree of hydrolysis (DH, %) were determined by the amine nitrogen (AN)/total nitrogen (TN) ratio in the final hydrolysate where AN is the amine nitrogen content determined by the method of formaldehyde titration (Lahl and Braun, [14]), and TN is the content of total nitrogen, determined titrimetrically by the Kjeldahl method.

*- Dry matter*

The amount of dry matter was determined by means of an Ohaus MB 25 (Ohaus Corporation, USA) moisture meter weighing (PRC) at 105 $^0$C.

*- Ion analysis*

The ionometry was carried out using a S400-B pH meter (Mettler Toledo, Spain).

2.2.2 Protein and large peptide

Qualitative analysis for proteins and large peptides was carried out by reaction with sulfosalicylic acid.

*2.2.2.1 Peptides*

Peptide concentration (PC) was determined using the biuret method (Gornall *et al.*, [9]) with 1% pepton as standard. Absorbance of the samples was measured at 540 nm in triplicates using UV spectrophotometer SF 102 (LLC «NPO Interfotofizika», Russia).

*2.2.2.2 Free amino acids composition*

Analysis of free amino acid (FAA) composition was carried out with automatic amino acid analyzer Aracus (Abacus, Germany).

*2.2.2.3 Sugars*

The amount of sugars in the hydrolysates was determined by the reaction with picric acid (according to Creselius-Seifert), using an SF 102 UV spectrophotometer.

2.2.3 Determination of antioxidant activity of the hydrolysates

*2.2.3.1 ABTS radical scavenging activity*

ABTS radical antioxidant activity was determined according to the procedure described by Metelitza *et al.*, [23]. In brief, the working solution was prepared by dissolving the chromogen containing $ABTC^+$ in 20 mL of Tris-HCl buffer. To 1.98 mL of the working solution was added 0.02 mL of hydrolysate, held for 3 minutes and the optical density was measured at a wavelength of 734 nm. As a standard, a solution of trolox was used. In the negative control distilled water was used instead of the hydrolysate. Analyses were carried out by using UV spectrophotometer SF 102. ATBS radical scavenging activity was expressed as TEAC (trolox equivalent antioxidant capacity) value.

*2.2.3.2 Lipid peroxidation inhibition assay*

The lipid peroxidation inhibition activity of the purified peptide was measured in an emulsion system according to the method described by Qian *et al.,* [28] where linoleic acid was replaced by oleic acid.

*2.2.3.3 Determination of the total antioxidant capacity by the electrochemical method*

The total antioxidant capacity was determined by the electrochemical method to accordance to Piljac-Žegarac *et al.*, [27] and Hoyos-Arbelaez *et al.*, [10]. The total antioxidant capacity was determined by using a "Tsvet-Yauza-01-AA" liquid chromatograph equipped with an amperometric detector (Russia). Gallic acid was used as reference.

2.2.4 Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry

Mixed hydrolysate (MHpancreatin2), which demonstrated the most beneficial qualities from the point of view of further study, was subjected to proteomic analysis using MALDI-TOF mass spectrometry. The hydrolyzate was centrifuged at 10,000 rpm for 4 minutes (MiniSpin microcentrifuge (Eppendorf AG, Germany). The supernatant (1 microliters) was deposited on the MALDI plate. Pretreated and untreated samples were overlaid with 1 microliters of matrix solution (saturated solution of a-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid). The matrix sample was cocrystallized by air drying at room temperature. Measurements were performed with a Microflex mass spectrometer (Bruker Daltonik, Bremen, Germany) using Daltonics FlexControl software (version 3.3.64). Spectra were recorded in the positive linear mode (laser frequency, 60 Hz; ion source 1 voltage, 19.4 kV; ion source 2 voltage, 17.3 kV; lens voltage, 9.1 kV; mass range, 0 to 20,000 Da). The internal calibration was performed using of the mass test standard MBT (Bruker Daltonics, Germany). For each spectrum 4000 shots from different positions of the target spot (automatic mode) were collected and analyzed. Protein identification was performed using the BIOPEP database (Minkiewicz P. *et al.*, [24]).

2.2.5 Statistical analysis

All statistical analyses were performed using the GraphPad Prism for Windows, version 5.0 (GraphPad Software, San Diego, CA, USA). Results were expressed as mean ± standard deviation. Statistical analysis was performed by Student's t-test. $P < 0.05$ was considered statistically significant.

## 3. Results and Discussion

### 3.1 Technology of hydrolysates and their basic physicochemical parameters

Analysis of the seven technological preparation of hydrolysate of the embryonic tissues shows that after centrifugation of EH, MHpepsin and MHt hydrolysate, a persistent proteinaceous slurry forms, complicating the subsequent purification process by micro and ultrafiltration. Presence of turbidity occurs for the low efficiency of these proteolysis methods due to the retention of proteins with high molecular weight. This increases the costs due to time and filters expenses, thus reducing the level of product yield.

For what concern AH and MHt hydrolysates, qualitative analysis with sulfosalicylic acid shows the presence of proteins. That imposes restrictions on the use of these hydrolysates parenterally. The presence of protein smaller than 10 kDa is expected since an ultrafiltration membrane with 10 kDa cutoff was used.

The basic physiochemical parameters characterizing the hydrolysates are reported in Table 1.

It can be seen that for what concerns total nitrogen, amine nitrogen and amount of peptide MHpp, MHpancreatin2 and MHpancreatin4 hydrolysates present similar and higher results with respect the others.

The pH of all hydrolysates was fallen within the limits, which corresponds to the most stringent standards for such substances, including agents for parenteral administration.

A low level of hydrolysis with trypsin is consistent with the data obtained by Chay Pak Ting *et al.*, [5]. Mixed hydrolysates using pancreatin (N.3 and N.4) were practically not different to the hydrolysate MHpp (mixed hydrolysate N. 1) in terms of total nitrogen. However, the level of amine nitrogen and number of peptide in MHpancreatin N. 4 with respect to MHpancreatin2 was more than 5.5%, and 4.7%, respectively. These data indicate that when large concentrations of pancreatin (4 mg/mL) are used in mixed hydrolysates, not only separation of complex proteins to amino acids occurs, but also additional fragmentation of low molecular weight peptides.

Finally, it is evident, by considering the hydrolysis technology complexity and raw materials cost, that basic physical chemical parameters of hydrolysates show that combined method of hydrolysis with 35% HCl and pancreatin (MHpancreatin2) is the most effective one.

### 3.2 The content of amino acids and other hydrolysis products

The MHpp sample was used as reference for comparison with the studied hydrolysates chemical composition. Technology of this hydrolysate preparation involves maximal number of steps, including acid and enzymatic (pepsin and pancreatin) hydrolysis.

**Table 1. Basic physicochemical parameters of hydrolysates, mean ± SD (n = 10)**

| Investigated parameters | AH | EH | MHpp | MHpepsin | MHpancr.2 | MHpancr.4 | MHt |
|---|---|---|---|---|---|---|---|
| Amount of dry matter, g/L | 22.4 ± 1.2 | 30.6 ± 1.1 | 39.5 ± 0.9 | 33,1 ± 0,9 | 35.2 ± 0.8 | 36.5 ± 1.4 | 25.9 ± 0.5 |
| Ionometry (pH) | 6,86 ± 0,08 | 7.15 ± 0.06 | 7.23 ± 0.06 | 7,28 ± 0,06 | 6.82 ± 0.06 | 6.90 ± 0.06 | 7.05 ± 0.08 |
| Reaction with sulfosalicylic acid on proteins | positive | negative | negative | negative | negative | negative | positive |
| Total nitrogen (TN), % | 0.23 ± 0.01 | 0.34 ± 0.01 | 0.39 ± 0.02 | 0,29 ± 0,01 | 0.37 ± 0.02 | 0.39 ± 0.02 | 0.26 ± 0.01 |
| Amine nitrogen (AN), mg% | 68.6 ± 2.8 | 97.4 ± 4.5 | 135.1 ± 7.5 | 77,3 ± 3,3 | 114.1 ± 5.8 | 142.5 ± 6.1 | 66.2 ± 1.9 |
| Degree of hydrolysis (DH), % | 29.8 ± 1.9 | 28.7 ± 1.5 | 34.6 ± 2.1 | 26,7 ± 2,0 | 30.8 ± 2.2 | 36.5 ± 1.8 | 25.5 ± 1.3 |
| Amount of peptides, mg% | 940 ± 88 | 1330 ± 90 | 1485 ± 98 | 1265 ± 50 | 1555 ± 25 | 1485 ± 87 | 1250 ± 40 |
| Amount of monosaccharides in recalculation for glucose, % | 0.14 ± 0.01 | 0.10 ± 0.01 | 0.17 ± 0.02 | 0,15 ± 0,02 | 0.18 ± 0.02 | 0.19 ± 0.02 | 0.15 ± 0.02 |

**Table 2. Amount of amino acids and other hydrolysis products in the hydrolysates, µg/ml, mean ± SD (n = 10)**

| Amino acids and other the hydrolysis products | AH | EH | MHpp | MHpepsin | MHpancr.2 | MHpancr.4 | MHt |
|---|---|---|---|---|---|---|---|
| Aspartic acid (Asx[1]) | 509.8 ± 13.4 | 126 ± 3.3 | 737.3 ± 18.9 | 700.6 ± 17.5 | 739.2 ± 19.4 | 730.1 ± 18.7 | 713.8 ± 17.8 |
| *Threonine (Thr) | 26.9 ± 0.7 | 91.4 ± 2.3 | 104.9 ± 2.6 | 49.1 ± 1.3 | 97.7 ± 2.44 | 134.8 ± 3.5 | 60.6 ± 1.6 |
| Serine (Ser) | 45.7 ± 1.2 | 126.4 ± 3.2 | 169.7 ± 4.2 | 87.3 ± 2.3 | 168.4 ± 4.7 | 217 ± 5.6 | 110.9 ± 2.8 |
| Glutamic acid (Glx[2]) | 95.9 ± 2.5 | 184.4 ± 4.8 | 431.5 ± 11.9 | 194.4 ± 5.4 | 298.1 ± 7.5 | 444 ± 11.1 | 207.6 ± 5.8 |
| Glycine (Gly) | 21.8 ± 0.6 | 30.3 ± 0.8 | 63.9 ± 1.63 | 33.4 ± 0.9 | 57.4 ± 1.6 | 66.8 ± 1.7 | 47.2 ± 1.3 |
| Alanine (Ala) | 31.1 ± 0.8 | 126.8 ± 3.2 | 139.3 ± 3.7 | 59.2 ± 1.6 | 126.5 ± 3.3 | 173.7 ± 4.5 | 72.4 ± 1.8 |
| *Valine (Val) | 20.1 ± 0.5 | 145 ± 3.7 | 126.3 ± 3.2 | 36.2 ± 0.9 | 123.2 ± 3.2 | 195.4 ± 5.1 | 46.2 ± 1.2 |
| Cystine (Cys) | 0.7 ± 0.01 | nd | nd | 0.5 ± 0.01 | nd | nd | nd |
| *Methionine (Met) | 12.7 ± 0.3 | 146.4 ± 3.8 | 116.4 ± 3.2 | 24.3 ± 0.6 | 73.7 ± 1.9 | 99.6 ± 2.7 | 24.9 ± 0.7 |
| *Isoleucine (Ile) | 17.2 ± 0.4 | 84.2 ± 2.1 | 86.1 ± 2.2 | 20.8 ± 0.5 | 75.4 ± 1.9 | 115.4 ± 3.2 | 30.4 ± 0.8 |
| *Leucine (Leu) | 31.2 ± 0.8 | 568.8 ± 15.4 | 525 ± 14.6 | 74.3 ± 1.9 | 431.5 ± 10.8 | 503.4 ± 13.6 | 75.3 ± 1.9 |
| Tyrosine (Tyr) | 24.8 ± 0.6 | 537.3 ± 13.8 | 490.5 ± 13.3 | 57.6 ± 1.6 | 468.1 ± 12.3 | 549.9 ± 14.5 | 70.6 ± 1.8 |
| *Phenylalanine (Phe) | 22 ± 0.5 | 627.3 ± 16.1 | 588 ± 14.7 | 75.3 ± 1.9 | 455.4 ± 11.9 | 553.6 ± 14.6 | 60.4 ± 1.6 |
| Histidine (His) | 108.2 ± 2.7 | 288.7 ± 7.8 | 332.9 ± 8.5 | 234 ± 5.8 | 254.1 ± 6.8 | 239.1 ± 5.9 | 209.9 ± 5.5 |
| *Tryptophan (Trp) | 51.4 ± 1.3 | nd | 5.1 ± 0.1 | 13.1 ± 0.4 | 5.9 ± 0.1 | 11.4 ± 0.3 | 19.9 ± 0.5 |
| *Lysine (Lys) | 40.5 ± 1.1 | 282.5 ± 7.8 | 416.6 ± 11.6 | 61.7 ± 1.5 | 398.1 ± 11.1 | 458.5 ± 12.1 | 90.9 ± 2.5 |
| Arginine (Arg) | 31.5 ± 0.8 | 445.5 ± 12.7 | 589.2 ± 15.5 | 46.5 ± 1.3 | 471.4 ± 12.1 | 747.5 ± 20.8 | 79.2 ± 2.1 |
| Proline (Pro) | 67.8 ± 1.8 | 7.1 ± 0.2 | 33.9 ± 0.9 | 15.8 ± 0.4 | 12.4 ± 0.3 | 25.8 ± 0.6 | 11.9 ± 0.3 |
| Phosphoserine (P-Ser) | 14.8 ± 0.4 | 35.2 ± 0.9 | 56.6 ± 1.6 | 27.9 ± 0.7 | 51.4 ± 1.3 | 16.9 ± 0.5 | 36.2 ± 1 |
| Taurine (Tau) | 21.3 ± 0.5 | 29.1 ± 0,8 | 41.3 ± 1.1 | 45.9 ± 1.2 | 54.8 ± 1.4 | 26 ± 0.7 | 55.3 ± 1.4 |
| Urea | 11.2 ± 0.3 | 61.3 ± 1.5 | 237.8 ± 6.4 | 186.9 ± 5.1 | 306 ± 7.8 | 885.8 ± 22.7 | 199.6 ± 5.3 |
| α -aminoadipic acid (a-AAA) | 2.1 ± 0.1 | 26.9 ± 0.7 | 9.3 ± 0.2 | 5.2 ± 0.1 | 10.8 ± 0.3 | 15.7 ± 0.4 | 5.3 ± 0.1 |
| Citrulline (Cit) | 0.9 ± 0.02 | 35.7 ± 0.9 | 12.4 ± 0.3 | 1.1 ± 0.03 | 6.4 ± 0.2 | 11 ± 0.3 | 1.3 ± 0.03 |
| α-aminobutyric acid (a-ABA) | 1.2 ± 0.03 | 2.6 ± 0.06 | 14.3 ± 0.4 | 1.5 ± 0.04 | 7.3 ± 0.2 | 6.5 ± 0.2 | 2.1 ± 0.05 |
| Cystathionine (Cystha) | 12.4 ± 0.3 | 29.9 ± 0.8 | 78.1 ± 2.1 | 20 ± 0.6 | 52.5 ± 1.4 | 52.5 ± 1.5 | 24.9 ± 0.6 |
| H-Cystine | 7.4 ± 0.2 | 13.1 ± 0.3 | 39 ± 0.9 | 1.2 ± 0.03 | 3.1 ± 0.1 | 15 ± 0.4 | 2.1 ± 0.1 |
| Gamma-aminobutyric acid (g-ABA) | 43.3 ± 1.2 | 11.3 ± 0.3 | 18.8 ± 0.5 | 145.6 ± 4 | 234.2 ± 5.9 | 198.5 ± 5.4 | 168 ± 4.2 |
| 1Methylhistidine (1Mehis) | 31.4 ± 0.9 | 176.5 ± 4.9 | 152.4 ± 4.2 | 26.9 ± 0.7 | 119.9 ± 2.9 | 172.5 ± 4.4 | 39.4 ± 1.1 |
| Carnosine (Car) | 70.3 ± 1.7 | 959.4 ± 23.9 | 631.7 ± 16.6 | 123.7 ± 3.3 | 490.3 ± 12.3 | 682 ± 18.9 | 146.5 ± 3.8 |
| Anserine (Ans) | 85.6 ± 2.1 | nd | nd | nd | 24.5 ± 0.6 | nd | 30.2 ± 0.8 |
| Hydroxylysine (Hylys) | 8 ± 0.2 | 140.5 ± 3.7 | 18.1 ± 0.5 | 4.6 ± 0.1 | 1.8 ± 0.1 | 3.2 ± 0.1 | 5 ± 0.1 |
| Ornithine (Orn) | 10.5 ± 0.3 | 18.8 ± 0.5 | 25.1 ± 0.7 | 10.3 ± 0.3 | 21.2 ± 0.6 | 12.4 ± 0.3 | 14.4 ± 0.4 |
| Ammonia ($NH_3$) | 89.3 ± 2.3 | 60.2 ± 1.5 | 130.7 ± 3.3 | 122.7 ± 3.2 | 136.3 ± 3.7 | 128.4 ± 3.2 | 111.6 ± 2.9 |
| Ethanolamine ($EOHNH_2$) | 19.35 ± 0.5 | 15.3 ± 0.4 | 35.1 ± 0.9 | 28.5 ± 0.7 | 35.9 ± 0.9 | 25.3 ± 0.7 | 40.2 ± 1.1 |
| Hydroxyproline (Hypro) | 1935.5 ± 48.4 | 15.1 ± 0.4 | 1569 ± 39.2 | 1562.1 ± 39.1 | 1632.5 ± 45.3 | 444.6 ± 11.7 | 1489.1 ± 37.2 |

Legend: [1]Asx comprise Asp +Asn; [2]Glx comprise Glu + Gln; *Essential amino acid; nd: not detected.

In all the hydrolysates obtained there is a wide list of amino acids (Table 2).

However the poorest in composition were AH, MHpepsin and MHt hydrolysates. The total number of amino acids in these hydrolysate compared to MHpp were less than 23%, 36% and 39%, respectively.

The amino acid composition founded in MHpp, EH, MHpancreatin2 and in MHpancreatin4 was also very different. The concentration of amino acids in EH hydrolysate, MHpancreatin2 and MHpancreatin4 in comparison with total quantity of amino acids in MHpp hydrolysate is equal 77%, 85.9% and 106.2% respectively. The analysis of amino acid profile of EH hydrolysate showed high concentration of Val, Met, Leu, Tyr and Phe. MHpancreatin4 hydrolysate is also characterized by high concentration of Thr, Ser, Ala, Val, Ile, Tyr, Lys and Arg. The concentration of a most of amino acids in MHpancreatin2 hydrolysate is not significantly different from MHpp ($P > 0.05$).

Analysis of the content of amino acids derivatives and other hydrolysis products in hydrolysates indicates that hydroxyproline is the most predominant compared with MHpp. The concentration of this amino acids derivative equal to 123.4% in AH sample and 104.1% in MHpancreatin2 sample. While in EH and MHpancreatin4, a high carnosine content of 151.9% and 108% was found. It is remarkable that concentration of gamma-aminobutyric acid in MHpepsin, MHpancreatin2, MHpancreatin4 and MHt hydrolysates is significantly higher than in MHpp.

The high carnosine and gamma-aminobutyric acid content in the extracts of chiken embryonic tissues is consistent with the results of Li *et al.*, [17] and may account for their high antistress, immunomodulating and antioxidant activity (Kim *et al.*, [12]; Kojima *et al.*, [13]; Meng *et al.*, [21]). The high content of hydroxyproline as the main product of collagen hydrolysis can cause high activity of hydrolysates for the repair of damaged tissues (Table 2).

The most well-balanced amino acid profile and composition of other hydrolysis products was found in MHpancreatin2. The main results of hydrolysis are the splitting of proteins with high molecular weight and recovery of specified peptides from chicken embryo tissues. The last phenomenon was shown on the case of carnosine and anserine.

### 3.3 Antioxidant activity of hydrolysates

All the hydrolysates obtained show a high antioxidant activity in TEAC analysis and range from 1.84 to 2.51 μM/mL. However, TEAC index of hydrolysates AH, EH and MHt was significantly lower than of hydrolysates MHpp, MHpepsin, MHpancreatin2 and MHpancreatin4 ($P < 0.05$) (Figure 2).

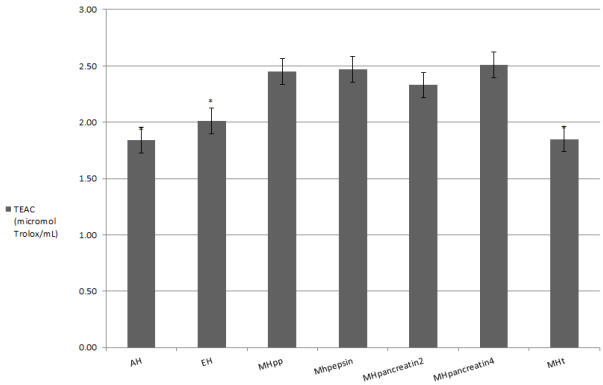


**Figure 2. ABTS radical scavenging activity of hydrolysates. ATBS radical scavenging activity was expressed as TEAC (trolox equivalent antioxidant capacity) value. Experiments were carried out in triplicate, and the expressed as mean ± SD and statistical differences between MHpp and other hydrolysates are indicated (*P < 0.05)**

In an attempt to establish the relationship between the antioxidant activity of various extracts and their amino acid composition, the content of various hydrolysis products was separately taken into account (Table 2). This results are in good agreement with data obtained by Chay Pak Ting *et al.*, [5], who calculated the total content of the specific (basic, acidic, hydroxylated, aromatic) or known antioxidant amino acids (pooled content 1 - Met, Tyr, Phe, His, Leu, Pro; pooled content 2 - His, Lys, Met, Leu, Phe, and Arg). These calculations showed that there is no significant relationship between the content of individual groups of compounds and their antioxidant activity.

The concentration of above listed specific amino acids in MHpepsin hydrolysate is significantly less than in MHpp, MHpancreatin2 and MHpancreatin4 hydrolysates. However, this effect has no significant influence on TEAC antioxidant activity of hydrolysates. On the other side, enzymatic hydrolysate (EH) characterized by higher concentration of specific (basic, hydroxylated, aromatic) and known antioxidant amino acid and statistically reliable lower TEAC antioxidant activity in comparison with the MHpepsin sample.

This may be due to the content of specific peptides in the hydrolysates, what is consistent with chemical data and the results obtained by Chay Pak Ting *et al.*, [5], Davalos *et al.*, [6], Duan *et al.*, [7], Kojima *et al.*, [13] and Nagasawa *et al.*, [25].

The hydrolysates were also evaluated for their antioxidant activity using the lipid peroxidation inhibition assay. As shown in Figure 3, all seven hydrolysates can act as significant inhibitors of lipid peroxidation, since they showed a significantly higher inhibition rate than trolox.

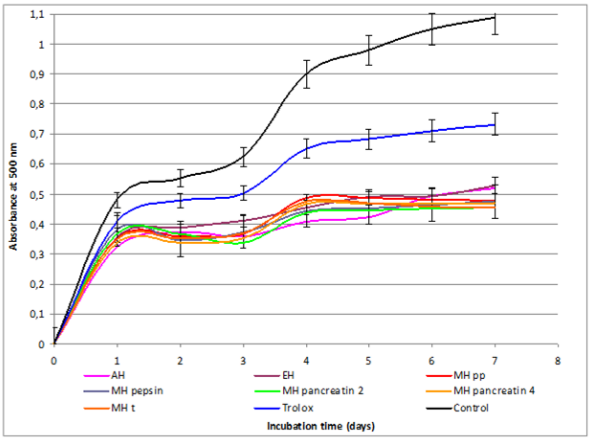


**Figure 3. Lipid peroxidation inhibition of hydrolysates. The activity was measured in oleic acid emulsion system by ferric thiocyanate method (FTC). Lower absorbance at 500 nm represents higher lipid peroxidation inhibition. Experiments were carried out in triplicate, and the expressed as mean**

Hydrophobic properties of the hydrolysates or peptides play an important role in quenching lipid derived radicals (Je *et al.*, [11]). Taking in account that total number of hydrophobic amino acids (Val, Ile, Leu, Pro, Met, and Phe) varied significantly from 171 µg/mL (AH) to 1578.8 µg/mL (EH) but lipid peroxidation inhibition level didn't changed, it is possible to conclude that specific peptides are main active substances, which neutralize lipid derived radicals.

In addition, all seven hydrolysates showed a high level of total antioxidant capacity from 3.09 to 10.44 mg/L using the electrochemical analysis method. The highest antioxidant capacity is found in hydrolysates EH, MHpp, MHpancreatin2 and MHpancreatin4 (from 7.76 to 10.44 mg/L per gallic acid), the lowest antioxidant capacity in hydrolysates AH, MHpepsin and MHt (from 3.09 to 4.65 mg/L per gallic acid) ($P < 0.05$).

It is remarkable that enzymatic hydrolysate (EH) showed the highest antioxidants activity, detected by electrochemical method and while is one of the lowest parameters when the TEAC method is applied. Conversely, MHpepsin sample was characterized by confusion high level of antioxidant activity by TEAC, but a lower antioxidant activity determined by electrochemical method. This may be connected with high concentration of low molecular substances (Met, Tyr, Phe, His, Leu, Pro, Car) in the composition of EH. Antioxidant activity of these substances is detected effectively by electrochemical method. These results are not unusual when comparing different methods for measuring the antioxidant activity. In fact, in the TEAC method trolox is used as reference, while the potential in the electrochemical method. Herewith total quantity of peptides in MHpepsin is compatible with the same parameter of EH. This fact me be explained by the presence of specific amino acid sequences with high antiradical activity (detected by TEAC) in MHpepsin composition.

All the hydrolysates obtained have antioxidant activity, but the highest levels were recorded in mixed hydrolysates produced with use of pancreatin (MHpp, MHpancreatin2 and MHpancreatin4). Taking into account hydrolysis technology complexity and raw materials cost, combined method of chicken embryonic tissues hydrolysis with application of HCl and pancreatin (MHpancreatin2) is recommended for industrial implementation.

### 3.4 MALDI-TOF mass spectrometry

The study of the MHpancreatin2 hydrolyzate showed that the obtained mass spectra include signals of different intensities in the range of 200 - 5000 Da (Figure 4).

In this case, a number of characteristic features are distinguished. Thus, about 15 signals were detected with

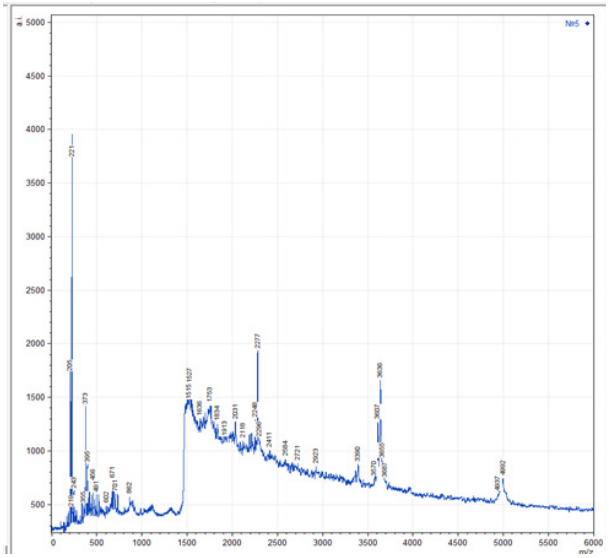

**Figure 4. Data of the MALDI-TOF mass spectrometry of the MHpancreatin2 hydrolyzate**

m/z up to 900 Da, about 20 signals are in the range of 1500 - 3700 Da and a small number of signals in the range of 4900 - 5000 Da.

Analysis of the obtained data using the BIOPEP database showed that the MHpancreatin2 hydrolyzate contains peptides that possess primarily antioxidant activity, ACE inhibitor activity, dipeptidyl peptidase IV inhibitor activity, and also antibacterial action (Table 3).

At the same time, peptides with antioxidant activity, ACE inhibitor activity, dipeptidyl peptidase IV inhibitor activity are concentrated in the region up to 900 Da, and peptides with antibacterial action are concentrated up to 2600 Da, more than this mass there is no correspondence of signals with biologically active peptides of the BIOPEP database was found.

**Table 3. Characterization of the proteomic analysis of the hydrolyzate MHpancreatin2 (in accordance with the BIOPEP database)**

| Chemical mass, Da | ID | Sequence | Activity |
|---|---|---|---|
| **243** | 3342 | GPA | ACE inhibitor |
| | 7810 | KP | ACE inhibitor |
| | 7837 | PQ | ACE inhibitor |
| | 8218 | KP | antioxidative |
| | 8500 | APG | dipeptidyl peptidase IV inhibitor |
| | 8519 | KP | dipeptidyl peptidase IV inhibitor |
| | 8522 | GPA | dipeptidyl peptidase IV inhibitor |
| | 8532 | QP | dipeptidyl peptidase IV inhibitor |
| | 8858 | PK | dipeptidyl peptidase IV inhibitor |
| | 8861 | PQ | dipeptidyl peptidase IV inhibitor |
| | 9041 | AGP | ACE inhibitor |
| **355** | 8000 | LHS | antioxidative |
| **373** | 7654 | NKL | ACE inhibitor |
| **395** | 8220 | TFE | antioxidative |
| **456** | 3620 | GRKP | immunomodulating |
| | 3776 | MPLP | antiamnestic |
| | 3779 | PPPF | dipeptidyl carboxypeptidase inhibitor |
| | 7652 | KFY | ACE inhibitor |
| **491** | 7904 | SALAM | antioxidative |
| | 7943 | YYF | antioxidative |
| | 7961 | FYY | antioxidative |
| | 7963 | YFY | antioxidative |
| | 8431 | MGSPT | antioxidative |
| | 8607 | WRM | dipeptidyl peptidase IV inhibitor |
| | 9070 | MRW | ACE inhibitor |
| **602** | 8278 | VPYPQ | antioxidative |
| | 8963 | VPVTST | antioxidative |
| **671** | 3367 | GKKVLQ | ACE inhibitor |
| | 9099 | MTEEY | ACE inhibitor |
| | 9100 | MTEEY | antioxidative |
| | 9109 | LIWKL | ACE inhibitor |
| **701** | 8306 | SVMPVVA | antioxidative |
| **862** | 9250 | VPSERYL | ACE inhibitor |
| **1515** | 2980 | KKAMRRQEAVDAL | kinases inhibitor |
| | 3893 | FFPVIGRILNGILG | antibacterial |
| **1527** | 3063 | QPTIPFFDPQIPK | immunomodulating |
| **1753** | 9240 | LVYPFPGPIPNSLPQN | ACE inhibitor |
| **1913** | 2596 | KWCFRVCYRGICYRRCRG | antibacterial |
| **2031** | 5516 | GXFGPAFHSVSNFAKKHKTA | antibacterial |
| **2584** | 3222 | GLLSVLGSVAKHVLPHVVPVIAEHL~ | antibacterial |

## 4. Conclusions

- In this study, various variants of hydrolysis of chicken embryo tissues were used to prepare peptide-based substances.

- Different methods of hydrolysates preparation have high influence on their antioxidant capacity. Selected amino acids and small peptides are responsible for their behavior. All hydrolysates showed antioxidant activity, but the highest values were recorded in mixed hydrolysates produced with use of pancreatin. This indicates the possibility of their use as food additives, pharmaceutical agents and as food components to prevent oxidative reactions.

- However, taking into account hydrolysis technology complexity and raw materials cost, combined method of chicken embryonic tissues hydrolysis with application of HCl and pancreatin (MHpancreatin2) is recommended for industrial implementation, as it contains peptides that primarily possess antioxidant activity, ACE inhibitor activity, dipeptidyl peptidase IV inhibitor activity, as well as antibacterial action.

- Further more detailed study and fractionation of peptides is required, as well as the study of their antioxidant activity, as well as other types of biological activity in vivo.

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