SUMMARY

Dry-cured pork loin proteins as a potential source of bioactive peptides - *in silico* and *in vitro* studies

Based on the review of the current state of knowledge, the following research hypothesis has been put forward: dry-cured pork loin proteins are a potential source of bioactive peptides.

The aim of this dissertation was to determine the potential of dry-cured pork loin proteins as precursors of biologically active peptides and to assess the possibility of its formation by using probiotic (*Lactobacillus rhamnosus* LOCK900; and *Bifidobacterium animalis* subsp. *lactis* BB-12) and potentially probiotic (*Lactobacillus acidophilus* Bauer Ł0938) strains of lactic acid bacteria as starter cultures.

Verification of such assumptions was made by three sub-goals: (I) determination of the influence of the time of ageing on the biological activity of peptides from dry-cured pork loins (II) determination of the effect of simulated digestion and absorption *in vitro* on the biological activity of peptides and (III) determination of the impact of probiotic and potentially probiotic strains of lactic acid bacteria on the biological activity of peptides from dry-cured loin.

The approach presented in the attached works consists in determining the relationship between the ageing process and the biological activity of peptides released during the twelve-month (360 days) ageing of pork loins. When designing the research hypothesis, it was assumed that the use of different strains of lactic acid bacteria may generate specific peptide sequences with antioxidant and the angiotensin I-converting enzyme inhibiting properties. Biologically active sequences were also evaluated during simulated digestion and *in vitro* absorption in the gastrointestinal tract. *In vitro* analyzes were preceded by a detailed literature review and *in silico* analysis.

Detailed characterization of biologically active meat-derived peptides and dry-cured meat products and the potential for their use in the context of functional food production are described in paper (**I**) titled "Meat and fermented meat products as a source of bioactive peptides". The paper presents the definition of biologically active peptides, describes the conditions of their formation with emphasis on their production and occurrence in fermented meat products and general characteristics of peptides in the context of their various bioactivity and possible health effects caused by their presence. Since, among the various biopeptides, angiotensin I-converting enzyme inhibitors and antioxidants are most commonly described, these two groups of biological activity of peptides were considered in further analyzes (**IV** and **V**).

In the work **II** and **III** *in silico* approach was shown (based on the database BIOPEP-UWM), wherein the selected pork muscle proteins were shown as a potential source of peptides with the selected activities, i.e. flavor precursors (**II**) and indicate their potential various biological activities (**III**). In study **II**, myofibrillar proteins have been identified

as crucial in shaping the sensory profile of dry-cured pork products. In particular, myosin-2 has proven to be a good precursor of peptides and flavor-active amino acids, most responsible for bitter, umami and acidic taste sensations. The role of peptides in suppression (acidity and sweetness) and enhancement (salty and umami) taste was also noticed, which was not observed in the case of amino acids. Troponin T was the only source of ingredients that inhibited sour taste.

The *in silico* analyzes presented in publication **III** were carried out in two stages. First, the potential of intact muscle proteins (as whole particles) was evaluated, and then the protein sequences were hydrolysed *in silico* with proteolytic enzymes, i.e. pepsin, trypsin and chymotrypsin (in one step). Based on the first part of the experiment, the presence of peptides with a total of 25 different potential biological activities present in the structure of the analyzed proteins was confirmed, which confirms the passage of high potential of meat and meat products to modulate the physiological functions of the human body. Of these, the enzyme inhibitory activity of dipetidyl peptidase IV and angiotensin I-converting enzyme inhibiting activity as well as antioxidant activity were most commonly determined (**III**).

Further studies on the *in silico* hydrolysis of protein sequences revealed that under the influence of the combination of digestive enzymes used, peptides potentially affecting the human organism may be formed, but the loss of peptide potential in the range of offered biological activity as a result of enzymes compared to intact protein sequences was observed. Among the studied myofibrillar proteins, myosin-2 and nebulin are the richest potential source of fragments demonstrating in particular the ability to inhibit the action of dipeptidyl peptidase IV and angiotensin I convertase after *in silico* hydrolysis. Myosin-2 was also a precursor to a large number of antioxidant peptides. It is also noted that more than one activity can be assigned to a given peptide sequence (**III**). These observations were verified by analyzes under the *in vitro* procedure.

An attempt was made to assess the biological activity of protein hydrolysates obtained from dry-cured pork loin (**IV** and **V**). The analyzes were carried out at various stages of the production process (i.e. after 28, 90, 180, 270 and 360 days of maturation). Protein extracts were also subjected to a two-step *in vitro* hydrolysis with pepsin and pancreatin and simulated absorption process using a semi-permeable membrane (7kDa). In addition, the obtained peptide fractions were subjected to chromatographic analysis with mass spectrometry, which allowed determining the amino acid sequence of the peptides. In the final step, the obtained sequences were analyzed in *in silico* approach and their potential biological activities were determined, assigning additionally peptide sequences to a particular protein. This approach made it possible to confirm or negate the results of *in silico* analyzes presented in publication **III** regarding the search for the source of the sequence modulating the functions of the human body.

In the present study, the sample with *L. acidophillus* Bauer Ł0938 showed the strongest antiradical activity (**IV**) within a fraction of WSF and the strongest inhibiting ACE activity within a fraction SSF (**V**). In view of the above, the use of the *L. acidophillus* Bauer Ł0938 strain is recommended as the most beneficial from the point of view of the release

of biologically active peptides at the product level and after the process of simulated digestion and absorption *in vitro* of dry-cured pork loins.

The influence of the time of ageing on the content of peptides (**IV**) in protein extracts and thus their biological activity was confirmed ($p < 0.01$) (publication **IV** and **V**). An increase in biological activity of extracts obtained from products after 180 days of ageing was observed, followed by a decrease in biological activity up to the end of the analyzed period, despite the progressing degradation of proteins. Based on this observation, the six-month production period was found to be the most optimal for the analyzed peptide activities.

Based on the results presented in works **IV** and **V**, the effect of the applied proteases on the biological activity of peptides resulting from the conversion of meat proteins was found. It has been shown that the progress of hydrolysis in simulated gastro-intestinal conditions promotes the release of particles with biological activity measured by the ability to capture the radical ABTS cations (**IV**) and acting as ACE (**V**) inhibitors. Nevertheless, when analyzing the various stages of digestion, fewer peptides with anti-radical properties were associated with pepsin digestion than with pancreatin (**IV**). It has been found that the level of ACE inhibition by hydrolysates after one-step digestion is higher than in the case of two-step digestion. The obtained results suggest that the specificity of the enzymes (**IV** and **V**), and not the degree of hydrolysis (**IV**), determines the release of bioactive peptides. Based on the results of chromatographic analysis of final hydrolysates (<7kDa), it was shown that 98% of identified peptides were a potential source of sequences indirectly responsible for lowering blood pressure (**V**), while only 38% of the sequences were identified as potential antioxidants (**IV**). Moreover, potentially the most active sequences acting as antioxidants were mostly derived from sarcoplasmic proteins. In turn, myofibrillar proteins were better potential precursors of ACE inhibitors than antioxidant peptides. Biologically active sequences with predicted ACE inhibitory potential were mainly derived from MLC1f protein (Uniprot ID: A1XQT6). These results are consistent with the trends obtained by *in silico* methods (**III**), thus confirming the importance of predictive methods for searching for sources of biologically active peptides.

Conclusions

1. The ageing time affects the biological activity of peptides from dry-cured pork loin. The six-month ageing period was considered the most optimal in the context of the analyzed biopeptide activities.
2. Simulated *in vitro* digestion affects the biological activity of peptides from dry-cured pork loin, the effect being different for the analyzed activities. The gastric digestion stage is the most preferred in the context of generating ACE inhibitor peptides.

In turn, the intestinal digestion step contributes to the release of peptides with strong antiradical activity.

3. Peptides obtained from dry-cured pork loin retain biological activity after the simulated absorption step *in vitro*.
4. A strain of *Lactobacillus acidophilus* Bauer Ł0938 is recommended for use in the production of dry-cured pork loin due to the high antioxidant activity and ACE-inhibiting effect of peptides obtained from products inoculated with this strain.

Reference to publications:

I. Stadnik Joanna, **Kęska Paulina** (2015). Meat and fermented meat products as a source of bioactive peptides. *Acta Scientiarum Polonorum Technologia Alimentaria*, *14*(3), 181 - 190.

II. **Kęska Paulina**, Stadnik Joanna (2017). Taste-active peptides and amino acids of pork meat as components of dry-cured meat products: An *in-silico* study. *Journal of Sensory Studies*, *32*(6), s. 1 - 9, art no.: e12301.

III. **Kęska Paulina**, Stadnik Joanna (2016). Porcine myofibrillar proteins as potential precursors of bioactive peptides - an *in silico* study. *Food & Function*, *7*(6)**,** 2878 - 2885.

IV. **Kęska Paulina**, Stadnik, Joanna (2018). Stability of antiradical activity of protein extracts and hydrolysates from dry-cured pork loins with probiotic strains of LAB. *Nutrients*, *10*(4), s. 1 - 15, art. no.: 521.

V. **Kęska Paulina**, Stadnik Joanna (2018). Ageing-time dependent changes of angiotensin I-converting enzyme-inhibiting activity of protein hydrolysates obtained from dry-cured pork loins inoculated with probiotic lactic acid bacteria. *International Journal of Peptide Research and Therapeutics*; https://doi.org/10.1007/s10989-018-9765-y.