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SYNERGISTIC EFFECT OF APPLICATION OF ENZYME PREPARATION WITH BIFIDOBACTERIA IN PROTEIN HYDROLYSATE MANUFACTURING TECHNOLOGY

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Abstract

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The purpose of the research was to study the applicability of an enzyme preparation with bifidobacteria. The technology of manufacturing a protein hydrolysate was developed. As a raw material second category by-products (lips and ears of cattle) were used. The action of temperature on the proteolytic activity of Protepsin is presented in the article, as well as the effect of pH level on the proteolytic activity of the enzyme preparation under consideration. When combining Protepsin and bifidobacteria, an increase in the amount of hydrolysis products – water-soluble proteins and peptides to 17.8 g/cm^3 is achieved.

Key words: enzyme preparation; bifidobacteria; protein hydrolysate; manufacturing technology; food biotechnology

Introduction

Supplying the country's population with protein-containing products is becoming a social issue, therefore using all food components of animal-derived raw materials to full advantage allows to increase the use of food resources, expand the range of food products, as well as to contribute to the solution of environmental problems (Arihara, 2006).

Secondary collagen-containing meat raw material contains a considerable quantity of food protein, cel has a physiological effect on the human body. Medical and biological studies have shown that the consumption of refined products free from ballast substances contributes to the development of lifestyle diseases such as atherosclerosis, diabetes, and osteochondrosis (PursIow, 2005).

The theory of adequate nutrition recognized in modern society suggests that when designing a diet it is most expedient to use functional ingredients, primarily those normalizing the microecological status of the lower gastrointestinal tract. The recommended ingredients are specially selected dietary fibers of different chemical composition and origin, non-enzyme antioxidants, microbial metabolites, probiotics and symbiotics (Dransfield, 1994).

Secondary meat resources include a considerable amount of offal, the yield of which is on average 18-22% of the live weight of the animal. Such meat by-products include:

- internal organs which during the life of the animal performed specific functions not associated with motor functions: liver, lungs, kidneys, brain, spleen, udder. Each of them consists of the core (base) and the parenchyma. The core is represented mainly by connective tissue, which includes nerves, blood and lymphatic vessels. The connective tissue core divides the organ into separate parts which contain the parenchyma (glandular tissue), responsible for the organ's basic function during life;
- organs which during the life of the animal wholly or partially performed motor functions: heart, diaphragm, tongue, stomach, trachea. Along with the connective tissue, they also contain muscle tissue (smooth or striated) (Rivier et al., 2007);
- external parts of animal carcasses: head, lips, legs, tail and ears, the structure and tissue composition of which are close to those of the meat carcass, differing in the quantity of muscle, connective and adipose tissue (Margander, 1995).

Of the listed raw materials, only 70% are used in traditional food production, the rest lacking the necessary nutri-

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tional values (lips, ears, manifold, spleen, trachea of cattle, rennet and lungs of small cattle) is used in the production of animal feed.

Food biotechnology is the fastest growing branch of biotechnology. One of the major areas for development of food biotechnology is the production and use of enzymes. Enzyme preparations for food production are obtained from organs and tissues of farm animals, cultivated plants (pineapple, soybean, papaya, fig) and special microorganism strains (Ashei et al., 2006). At present, enzymes of animal origin are primarily used.

Meat processing technology employs enzyme preparations with proteolytic, lipolytic, and collagenolytic activity, which are used for tendering, increasing the grade of meat as well as for production of protein hydrolysates (Mandl, 2000).

The importance of protein hydrolysates in meat processing is constantly increasing, which thereby significantly increases their role in human nutrition (Hammes et al., 2002).

Various methods of obtaining protein hydrolysates allow one to get products with specified properties (Ghaffari-Moghaddam et al., 2014).

Depending on the amino acid content and the presence of polypeptides in the range of the corresponding molecular weight, one can determine the area of the most effective use of hydrolysates.

Protein hydrolysates for the meat processing industry must meet two basic requirements: organoleptic properties and a balance in amino acid composition (Greco et al., 2005).

The demand of the industry in enzyme preparations is met by importing more than 90%. Russian manufacturers account for less than 10%, respectively. One of the enterprises that focus on food enzymes production is ZAO Endocrine Enzymes Plant, manufacturer of Protepsin enzyme preparation.

By nutrition value, by-products are divided into two categories depending on the collagen protein content. The first category includes liver, kidneys, brain, heart, beef diaphragm, beef and mutton meat and bone tails; the second category includes spleen, trimmings, beef udder, ears, trachea, rumens, rennet, legs, lips, ears and manifold; pork legs, tails and stomachs (Morgan et al., 1993).

Let us consider the characteristics of the second category cattle by-products. In the structure of by-products collagen proteins are distributed in the following way (% of the mass of raw tissue): tendons – from 25 to 35; cartilage – from 10 to 15; vessel walls – from 5 to 12; kidneys – from 0.4 to 1.0; liver – from 0.1 to 1.0; brain – from 0.2 to 0.4; tongue – 2.5; lungs – 3.3; spleen – 1.38; rumen – 6.8 (Neklyudov et al., 2007). In addition to the rumen, which contains 6.8% of collagen from the mass of the raw tissue, it is necessary to point out tendons in which 88.5% of the total protein mass is represented by collagens; ears and lips, containing respectively 77.2 and 75.7% of collagen from the total protein mass. Such by-products as ears, lips, rumens and udders contain a lot of collagen and elastin (Table 1) (Fayvishevsky, 2013).

Beef by-products of the second category according to the collagen content and, consequently, the potential protein source can be arranged in the following sequence: ears – lips – rumen – lungs (Wess, 2008).

Materials and Methods

The materials for the study were the following:

– second category cattle by-products with a high content of connective tissue (lips and ears);

– Protepsin enzyme preparation (with standard proteolytic activity of 100 units/g), manufactured by ZAO Endocrine Enzymes Plant in Rzhavki, Solnechnogorsk district, Moscow region;

– Leaven with active bifidobacteria (*B. bifidum* and B. Longum strains), bifidobacteria content is $1x10^9$ CFU / g.

Protepsin is an enzyme preparation of animal origin, containing a complex of acidic proteinases, intended for use in the meat industry for processing raw meat. The enzyme composition of the preparation is developed so as to have a balanced effect on various proteins of meat and meat systems used in the technology of meat processing. Protepsin's action in the meat system is similar to intracellular enzymes (cathepsins). It produces a synergistic effect and has additional qualities that allow to apply it in a wider range of technological parameters, and also to influence those protein systems

Table 1
Chemical composition of cattle by-products (according to M.L. Faivishevsky, 2013)

Raw material	Weight content, %				
	moisture	*ash*	*fat*	*total protein*	*collagen from total protein, %*
Rumen	76.1–80.0	0.5–0.6	4.1–4.2	14.8–17.1	61.0–62.3
Udder	72.6	0.3	13.7	12.3	52.0–54.0
Lips	73.7	1.4	3.4	20.8	75.0–76.0
Ears	60.9–69.8	1.4	2.3–14.1	21.1-25.2	77.0–89.2

Table 2
Protein hydrolysate formula

Raw material	Quantity of raw material, kg
A mixture of collagen-containing raw materials (lips and ears of cattle)	79
Protepsin enzyme preparation (with a proteolytic activity of 100 U/g)	0.2
Leaven with active bifidobacteria (*B. bifidum* and *B. Longum* strains), bifidobacteria content is $1\mathbf{x}10^9$ CFU/g	2
Preservative (sodium citrate)	0.8
Rice flour	4
Water	14

on which intracellular enzymes have an insignificant effect or do not have any effect at all (Spohner et al., 2015).

General characteristics of Protepsin are the following:

– it is a powder of light gray color,

– the standard preparation has three modifications with the proteolytic activity of 50, 100, 150 units/g.

The experimental formula of the protein hydrolysate is shown in Table 2.

The total proteolytic activity was determined using the modified Anson method with Hammerstein-grade casein at pH 7.2 as a substrate. One unit of proteolytic activity was considered equal to the amount of enzyme which per 1 minute at 30°C turned Hammerstein-grade casein in an amount corresponding to 1 μmol of tyrosine into the state in which it is not precipitated by trichloroacetic acid (Kuzelov et al., 2002).

Collagenase activity was determined by the content of hydroxyproline in the mixture formed as a result of the effect of the enzyme on native collagen. For this purpose, an oxidation reagent was prepared: 28.2 g of chloramine T was dissolved in 40 cm^3 of water to get a 0.05 M concentration, 60 $cm^3$of acetate citrate buffer with pH of 6.0 was added. To 2 cm^3 of the sample containing hydroxyproline 1 ml of the oxidation reagent was added, shaken and left for 20 minutes at room temperature. Then 1 cm^3 of 4 M perchloric acid was added to the mixture and shaken. 5 minutes later 3 cm^3 of a 10% solution of p-dimethylaminobenzaldehyde in methyl cellosolve was added. The sample was heated for 15 minutes in a water bath at 150°C and after cooling it was scanned using a green filter of the photoelectric colorimeter FEK-56M (555 nm).

The collagen hydrolysis was carried out under the following conditions: 20 mg of native collagen was treated with the enzyme preparation in the presence of a buffer system with pH of 7.0 so that the total volume was 25 cm^3. The mixture was incubated for 1 hour at 37°C. The mixture was controlled using samples incubated under the same conditions but without the enzyme, preliminarily stopping the reaction by adding 0.5 cm^3 of ethanol and centrifuging the mixture for 15 minutes at 6 000 rmp (Skurikhin et al. 1998).

All measurements were carried out in three replications. Statistical analysis was performed using Microsoft Excel XP and Statistica 8.0 software package. The statistical error of the data did not exceed 5% (at 95% confidence level).

Results and Discussion

Development of Technology for Protein Hydrolysate Manufacturing

Secondary collagen-containing raw material – lips and ears of cattle – taken in a ratio of 1:2 in the amount of 79 kg was washed, ground in the meat mincing machine and then processed in the cutter. After adding water and 0.2 kg of the enzyme preparation to the minced raw material, the resulting mixture was heated to 40–45°C. Hydrolysis was carried out for 1.5–2 hours. In order to inactivate the enzyme preparation the mixture was heated to the temperature of 80–90°C for 15–20 minutes and homogenized adding 2 kg of leaven with active bifidobacteria, rice flour and preservative according to the formula and then thoroughly mixing again.

Study of the Synergistic Effect of the Use of the Enzyme Preparation with Bifidobacteria

At the first stage of the study, the action of temperature on the proteolytic activity of Protepsin enzyme preparation was determined (Figure 1). To determine the dependence of optimum action of the enzyme preparation on the temperature, 2 ml of a 2% solution of a standard substrate were taken, 2 ml of solutions of homogeneous fractions of the proteolytic complex with a protein content of 600 μg / ml were added. Hydrolysis was carried out at a temperature of 0 to 90°C for 30 minutes. The temperature was maintained using a water thermostat. Similar research was conducted under the influence of pH on reaction mixtures.

Figure 1 shows that the extremal curve has the form of a "bell" with a clearly visible maximum, which can be explained by the high rate of avalanche-like thermal inactivation.

Protepsin shows maximum activity at moderate temperatures (35–40°C).

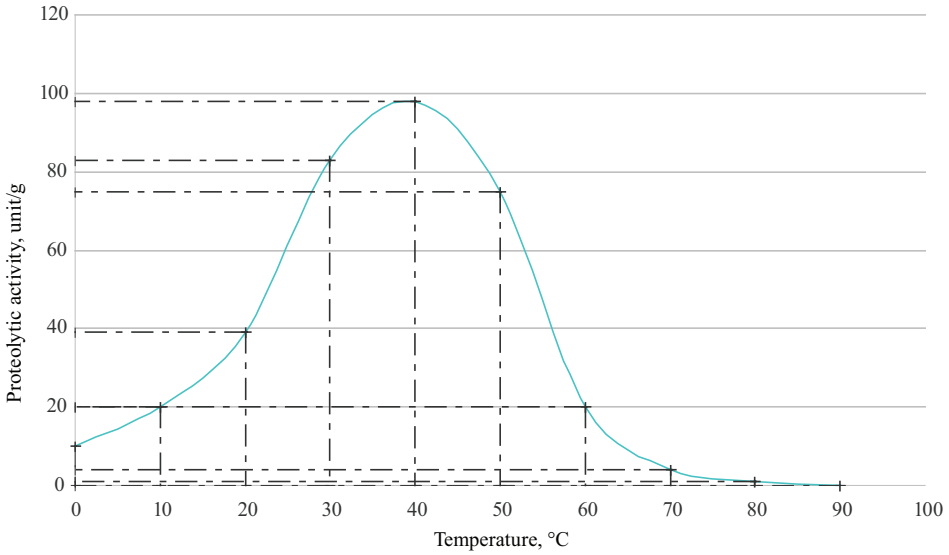

Fig. 1. Action of temperature on the proteolytic activity of Protepsin

As it is known, the temperature range of 0-4°C has the greatest practical value in the meat industry being the operation range for storage and processing of raw materials, in the first place for salting. At this temperature range Protepsin shows only about 15% of its maximum activity. Not least important is the high residual activity of this enzyme preparation at high temperatures (60–65°C), since this makes it possible to carry out an enzymatic reaction with the raw material during some types of heat treatment. In view of the fact that heat treatment is limited in time, the activity is critical. As is shown in the figure, Protepsin is almost completely inactivated at 70°C and above. The conducted research showed that after reaching 72°C (the temperature indicating that the product is cooked in most technologies), Protepsin is completely inactivated within 5 minutes.

The effect of pH level on the activity of the preparation was studied in the range of 2.0 to 10.0 at the temperature of 40°C.

The obtained results concerning pH effect on the proteolytic activity of Protepsin are shown in Figure 2.

As can be seen in the figure, Protepsin shows the highest proteolytic activity in the system in the pH range of 4.5–6.0.

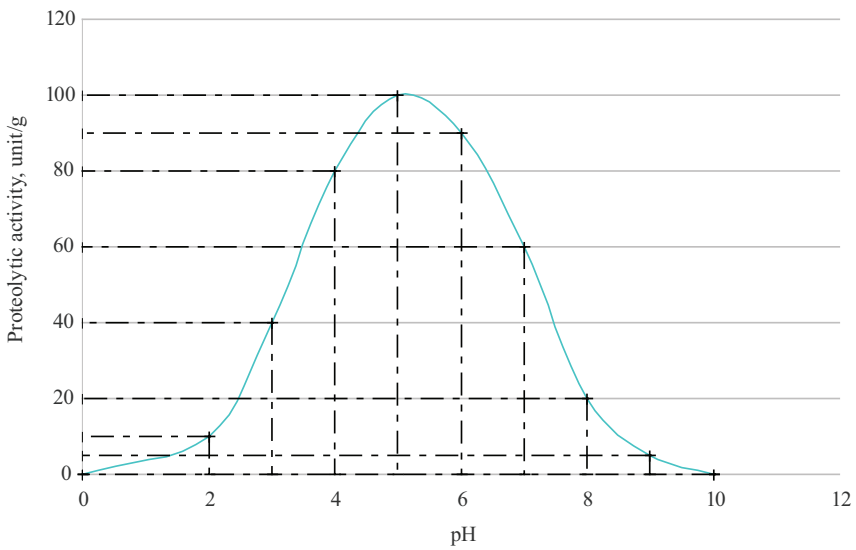

Fig. 2. Effect of pH on the proteolytic activity of Protepsin enzyme preparation

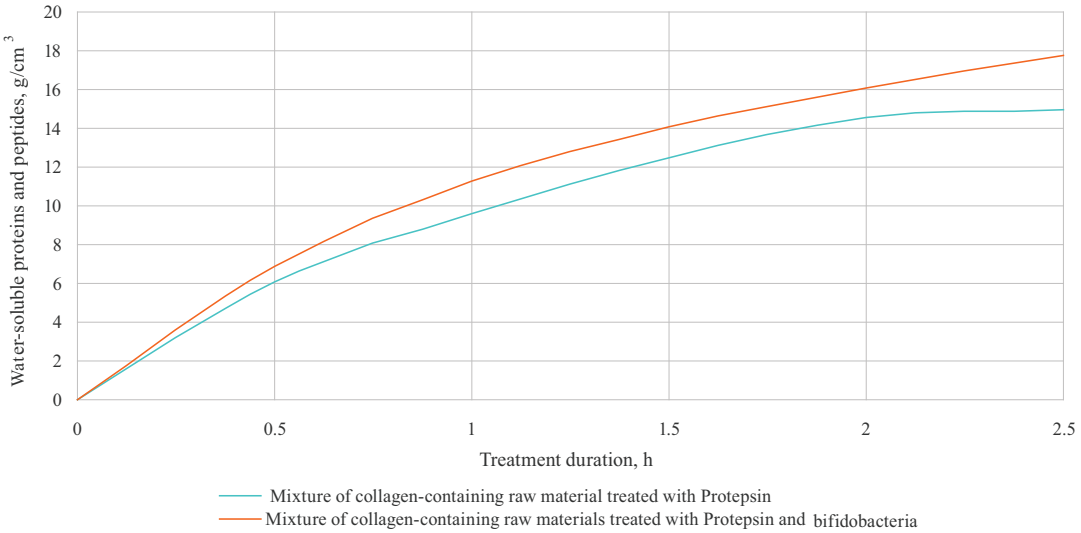

Fig. 3. Mixture of collagen-containing raw materials treated with Protepsin and bifidobacteria

This explains the choice of sodium citrate as one of the formula components. Besides its preservative action, sodium citrate shifts pH to the acidic side, thereby increasing the proteolytic activity as well as the product stability during storage. Sodium citrate enhances the activity of tissue enzymes and significantly increases the effectiveness of Protepsin.

The criterion for the efficiency of hydrolysis of concomitant protein fractions of albumins and globulins of collagen-containing raw material was the accumulation of hydrolysis products having peptide bonds in the liquid fraction of the hydrolysate (Terry et al., 1990).

The study was carried out using the enzyme preparation and a combination of the enzyme preparation with bifidobacteria (Figure 3).

According to Figure 3, when combining Protepsin and bifidobacteria, an increase in the amount of hydrolysis products – water-soluble proteins and peptides to 17.8 g/cm^3 is achieved (Morelli, 2000).

When the treatment of collagen-containing raw material with Protepsin lasts less than 1.5 hours, hydrolysis of the ballast protein fractions is insufficient; consequently, when the processed raw material is subsequently washed with water they are not removed, which adversely affects the quality of the protein hydrolysate.

An increase in the treatment duration to over 3 hours slightly affects the quantitative characteristics of proteolysis with the formation of water-soluble proteins and peptides, while simultaneously causing higher loss of the collagen fraction.

Adding rice flour to the formula increases the content of essential amino acids. Starch and dietary fiber are necessary for the nutrition and life of bifidobacteria.

At the final stage, an organoleptic evaluation of the protein hydrolysate was carried out. The results are shown in Table 3.

Table 3
Organoleptic evaluation of protein hydrolysate

Parameter	Characteristics
Appearance and consistency	Mixture of monolithic structure and smearing consistency
Colour	Dark pink with a grayish tinge
Smell	Characteristic of such by-products
Transparency	Nontransparent

The use of Protepsin in combination with bifidobacteria increases the degree of hydrolysis of collagen-containing raw material and allows producing a hydrolysate with a high degree of protein breakdown. The resulting protein hydrolysate can be further used for sausage manufacturing.

The article considers the possibility of obtaining protein hydrolyzate from secondary collagen-containing raw materials. A technology was developed for obtaining protein hydrolyzate from lips and ears of cattle using Protepsin enzyme preparation (with proteolytic activity of 100 U/g) and bifidobacteria (*B. bifidum* and *B. Longum* strains) with a concentration of 1x10^9 CFU/g. Lips and ears of cattle, taken in a ratio of 1:2 in the amount of 79 kg was washed, ground in the meat mincing machine and then processed in the cutter. After adding water and 0.2 kg of the enzyme preparation to the minced raw material, the resulting mixture was heated to 40–45°C. Hydrolysis was carried out for 1.5–2 hours. In order to inactivate the enzyme preparation the mixture was

heated to the temperature of 80–90°C for 15–20 minutes and homogenized adding 2 kg of leaven with active bifidobacteria, rice flour and preservative according to the formula and then thoroughly mixing again.

Sodium citrate was chosen as one of the formula components. Besides its preservative action, sodium citrate shifts pH to the acidic side, thereby increasing the proteolytic activity as well as the product stability during storage. Sodium citrate enhances the activity of tissue enzymes and significantly increases the effectiveness of Protepsin

Adding rice flour to the formula increases the content of essential amino acids. Starch and dietary fiber are necessary for the nutrition and life of bifidobacteria.

In the course of the experiments, it was found that Protepsin shows maximum activity at medium temperatures (35–40°C). Almost complete inactivation of the enzyme complex occurs at 70°C or higher within 5 minutes. Protepsin shows the highest proteolytic activity in the system with the pH range of 4.5–6.0. With the combination of Protepsin and bifidobacteria, the amount of hydrolysis products – water-soluble proteins and peptides – increases to 17.8 g / cm^3.

The application of biotechnological methods contributes to the creation of low-waste technologies, allows enhancing technological processes, improve and create a greater variety of finished products, reduce raw material consumption per unit of output, improve production standards and working conditions, as well as reduce pollution and amount of sewage.

Conclusions

In the course of experimental studies it was found that the preparation shows its working action at 20–45ºC, while the optimal temperature for the enzyme action in meat systems is 40ºC.

Complete inactivation of the enzyme complex occurs at 70°C within 15 minutes.

The proposed amount of the preparation that should be added is calculated for the state of the system with pH level of 4.5–6.0.

The introduction of bifidobacteria into the system has a synergistic effect on the enzyme preparation, promoting a deeper hydrolysis of collagen-containing raw material.

All the formula components are selected taking into account the physical and chemical parameters of the raw materials.

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