Safety assessment of \( \beta \)-lactoglobulin hydrolysate with reduced allergenicity

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Abstract. The increase in production of milk whey and the large-scale introduction of membrane technologies for fractionation of its various components has led to a significant interest in the use of whey proteins. Their application in food technologies is constrained by their potential antigenicity. The authors developed a technology for reducing the allergenicity of the \( \beta \)-lactoglobulin in the ultrafiltration concentrate of cheese whey by means of biocatalytic conversion using enzyme preparations Flavorpro 750MDP and Promod 439L. The purpose of the research was to justify the required modes of proteolytic enzymes inactivation in the obtained \( \beta \)-lactoglobulin hydrolysate that can ensure safety for a living organism. The mode of enzymes inactivation which is necessary and sufficient for reducing their activity in the hydrolysate when consumed was determined with a certain safety margin \((t = (80 \pm 2) \degree C, \tau = 5\) min). Preclinical studies of the obtained food composition were carried out by pharmacoetological method on adult BALB/c mice. Significant differences were found in horizontal and vertical motor activity, as well as the frequency of "looking into burrows" and the level of defecation. Under new conditions the animals of the experimental group demonstrated more exploratory behaviour rather than defensive one. This indicates a positive effect of \( \beta \)-lactoglobulin hydrolysate on the physical and psycho-emotional state of the tested animals and its safety for a living organism. The developed technology allows to obtain functional foods based on \( \beta \)-lactoglobulin hydrolysate with target physiological and biochemical properties, reduced allergenicity, increased food and biological value.

1. Introduction

The production of milk-intensive protein products (cheese, cottage cheese, technical casein) provides the formation of whey, which causes significant economic damage and irreparable harm to the environment [1]. The volumes of obtained whey reach 90 % of the volume of processed milk. In practice, they are slightly less due to incomplete collection and process losses. About 50 % of milk solids remain in the whey. The increase in production volumes of this raw material and the large-scale introduction of membrane technologies for fractionation of its various components have led to a significant interest in the use of whey proteins [2, 3].

The widespread use of these ingredients in food technologies is constrained by their residual antigenicity. According to the International Union of Immunological Society, the most common allergens in dairy products include caseins, immunoglobulins, \( \beta \)-lactoglobulin, \( \alpha \)-lactoalbumin and bovine serum albumin [4]. In this regard, the problem of making functional low- and hypoallergenic products containing a complete protein that does not cause allergic reactions becomes urgent [5 – 7].

The technology for reducing the allergenicity of the main whey protein - \( \beta \)-lactoglobulin - in the ultrafiltration concentrate of cheese whey has been developed [8]. For this purpose, the biocatalytic
conversion was used under the action of the enzyme preparations Flavorpro 750MDP and Promod 439L, which provides the production of a hydrolysate with a specified residual antigenicity (no more than 10%). \( \beta \)-lactoglobulin hydrolysate is characterized by appropriate organoleptic properties since it contains short-chain peptides with a controlled molecular-weight distribution, which do not have a bitter taste, and can be used in the technology of a wide range of dairy and fermented milk products for partial replacement of skimmed milk in the formulation, reducing the residual antigenicity of finished products and enriching them with minerals and vitamins.

2. The purpose of the study
The purpose of the research was to justify the modes of inactivation of the proteolytic enzymes Promod 439L and Flavorpro 750MDP in the \( \beta \)-lactoglobulin hydrolysate, which are necessary to provide its safety for a living organism.

To achieve this goal the following tasks were defined:
- to determine the temperature and duration of \( \beta \)-lactoglobulin hydrolysate pasteurization sufficient for satisfactory inactivation of the enzyme preparations used;
- to assess safety of the developed food composition for a living organism in preclinical studies.

3. The object of the study
Objects of the research were cheese whey concentrate with total protein in dry matter of 3.19 %, obtained using MMS Swissflow UF industrial ultrafiltration unit with ceramic membranes; \( \beta \)-lactoglobulin hydrolysate produced using proteolytic enzyme preparations Promod 439L and Flavorpro 750MDP.

4. Materials and methods
Hydrolysis of \( \beta \)-lactoglobulin in the cheese whey UF-concentrate was carried out in the fermenter for 6 hours at a mixer rotation frequency of 175 rpm and at the temperature recommended by the enzyme preparations manufacturer. The efficiency of the process was evaluated by mass fractions of total protein and whey proteins in the samples before and after hydrolysis in accordance with GOST 34454-2018 and GOST R 54756-2011.

An important aspect of applying protein hydrolysates obtained by proteolysis is the safety margin of the enzymes used. It was calculated on the basis of the dosage of enzyme preparations, the specified multiplicity of their activity decrease, and the amount of residual protein after inactivation.

Preclinical studies of the obtained \( \beta \)-lactoglobulin hydrolysate were carried out by ethopharmacological open field test, which is widely used to study behavioural reactions in pharmacology and psychogenetics and involves investigating the motor component of orientation response and emotional reactions of animals [9]. Sixty experiments were conducted on adult BALB/c mice. Experimental and control groups containing 30 individuals aged 15 days in each were formed for this purpose. The animals of the control group were kept on a diet according to GOST R 50258, consisting of a mixture of cereals in accordance with recommendations. The obtained \( \beta \)-lactoglobulin hydrolysate was introduced into a diet of the animals from experimental group.

All experiment procedures were carried out in accordance with International Standards for Care and Use of Laboratory Animals. Mice were kept in the vivarium under standard food and temperature conditions. The behaviour of the animals was observed in a special "open field" apparatus consisting of a white arena (62 cm diameter) surrounded by opaque walls (31 cm high) and divided into sectors, each intersection having a burrow (a hole in the floor). The unit was cleaned after each experiment. There was no special lighting of the arena, the tests were performed in the room lit by artificial daylight (~150 lux) in the evening with a noise level of about 20 dB. In these conditions, a number of elementary motor actions and postures were registered, which are characteristic to a conduct in the "open field". The testing study was carried out for 3 days in succession in order to objectify comparison parameters of behaviour and emotionality and avoid fragmentary evaluation.

Each animal was placed in the centre of the arena with its tail towards the researcher and observed for 5 minutes. The data was registered every time a mouse crossed the sector line. The number of crossings was recorded for each minute separately. Apart from horizontal activity (total number of line
crossings in the center and in the periphery), the open field test was used to register the frequency with which a mouse looks into a burrow (putting its head inside the hole), grooming (frequency of bringing front paws to the mouth and licking them, cleaning the front of the muzzle, body, as well as the duration of grooming), defecation (frequency of defecation) as well as vertical motor activity (frequency with which a mouse stands on its hind legs with and without support). The results were collected during 5-minute sessions, then the average value for 1 minute was calculated.

Statistical analysis of the results was performed using mathematical statistics according to data obtained from 5 to 10 experiments in three replications. The application package "MathCad" and "Microsoft Excel" were used for information processing and graphical interpretation of the results. The obtained results are characterized by high precision, interoperability of experimentally obtained values, and correct statistical processing.

5. Discussion of the results

Inactivation of the used enzymes is second in importance after protein proteolysis. The content of the active enzyme in the finished product should be next to zero which is usually achieved by high-heat treatment. In addition, it should have a minimal effect on the components of the obtained hydrolysate and does not cause changes in its chemical composition. Therefore, first of all a safety margin of proteases is calculated, which is then used to determine the required time for satisfactory inactivation of the enzymes.

It is known that the maximum quantity of the enzyme that provides “No-observed-adverse-effect level” (NOAEL) should not exceed 0.44 Anson units (AU)/kg/day [10].

The concentration of the enzyme preparation Flavorpro 750 MDP in Anson units in the UF-concentrate of cheese whey with 3.19% of total protein in dry matter is 940 AU/kg of protein. Consequently, a 1000-fold decrease in its activity will not be enough to ensure the safety for humans when using hydrolysate in food. With a 10,000-fold decrease in the activity of the enzyme preparation Flavorpro 750MDP, the safety margin will be: 0.44/0.094 = 4.68.

The initial concentration of the enzyme preparation Promod 439L in Anson units in the UF-concentrate of cheese whey with 3.19% of total protein in dry matter is in the range of 470 AU/kg of protein. With a 10,000-fold decrease in the activity the safety margin will be: 0.44/0.047 = 9.36.

The time necessary for proteases inactivation (min) for a tenfold decrease in their activity is calculated using the formula [11]:

$$t_D = 1.19 \times 10^{(75-T)/8.31} \cdot (1 + S \cdot 10^{(75-T)/94}) \text{, min},$$

where $T$ is inactivation temperature, °C;
$S$ is substrate concentration, %.

Thus, for the enzyme preparations used, the duration of inactivation by pasteurization at $t = (80 \pm 2)^\circ C$ for a tenfold decrease of activity will be:

$$t_D = 1.19 \times 10^{(75-80)/8.31} \cdot (1 + 3.18 \times 10^{(75-80)/94}) = 1.14 \text{ min}.$$ 

It has been proved, that decrease in enzyme preparations activity by 10,000 times requires heating for a longer time, at least 4 times relative to a tenfold decrease in activity [11]. Thus, 1.14 \cdot 4 = 4.56 min is sufficient for satisfactory inactivation of the enzymes preparations Promod 439L and Flavorpro 750MDP.

In order to assess the digestibility of the developed food composition and its safety for a living organism, studies were conducted on white inbred mice.

It is known, that in active animals, research motivation prevails over the emotion of fear of an unfamiliar environment. The open field test identified such types of exploratory behaviour as vertical (with or without support on the wall) and horizontal motor activity, "looking into burrows" as well as emotional manifestation (grooming and defecation).

The exploratory and motor activity of mice from the experimental group is higher than from the control one, and the level of defecation is lower, which may indicate a higher level of anxiety of mice from the control group (figure 1, 2). The most striking contrast was observed in vertical motor activity.
(figure 3) with the average rearings of $0.3 \pm 0.05$ per minute during the entire experiment for the mice from control group, compared to $0.8 \pm 0.3$ for the mice from the experimental group.

Figure 1. Total horizontal motor activity of mice in the centre and periphery of the arena

Figure 2. Frequency of "looking into burrows"

Figure 3. Vertical motor activity of mice

Figure 4. Average indicators of mice emotional activity

It is known from scientific literature [12, 13] that frequent and quick self-cleaning is an alarming grooming, and a high level of defecation further indicates the animal's anxiety and fear. A significantly
higher level of defecation in the control group of mice (figure 4) proves that they have a greater degree of anxiety, compared to the mice from the experimental group.

Thus, significant differences are observed between the mice from experimental and from control groups, both in horizontal motor activity, and in other parameters studied. Consequently, in new conditions the animals of experimental group showed more exploratory behaviour, rather than defensive. This indicates a positive effect of \(\beta\)-lactoglobulin hydrolysate on the physical and psycho-emotional state of the tested animals and its safety for a living organism.

6. Conclusion
The developed technology opens up new opportunities in solving the problem of complex, economically feasible and environmentally friendly processing of secondary dairy raw materials allowing to obtain functional food products based on the \(\beta\)-lactoglobulin hydrolysate with target physiological and biochemical properties, reduced allergenicity, increased food and biological value.

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