INTRODUCTION

Over 600 inherited metabolic disorders, including hereditary disorders of amino acid metabolism (histidinemia, phenylketonuria, tyrosinemia, alkaptonuria, and others), have been identified to date. Diseases associated with disturbed amino acid metabolism develop if the activity of an enzyme involved in the metabolism of ingested amino acids is selectively reduced. These diseases affect the central nervous system, resulting in mental retardation with motor and speech defects, impaired vision and hearing, emotional and behavioral disorders, and seizures [1, 4]. Hereditary metabolic disorders are caused by mutations introducing changes into the nucleotide sequence of genomic DNA. Mutations result in the synthesis of abnormal proteins (including structural proteins, enzymes, hormones, growth factors, and receptor proteins). For instance, if the mutation affects a gene encoding an enzyme, the latter loses its catalytic activity partially or completely [2, 21]. The vast majority of hereditary metabolic disorders is caused by genetic defects affecting enzymes involved in the metabolism of amino acids, carbohydrates, and lipids. Pathogenesis and clinical manifestations are determined by the lack of normal metabolites (intermediate or final) and the accumulation of toxic metabolites. The clinical presentation largely depends on the degree of the mutant gene expression, as well as on other genetic factors and environmental conditions. Mental retardation and neurological disorders are among the most frequent and severe consequences of the biochemical defect in the majority of patients [3, 5, 17].

Total screening for histidinemia, an innate defect of amino acid metabolism, in newborn babies has been recently introduced in Russia. Histidinemia is a hereditary disease associated with a disturbance of the metabolism of the amino acid histidine. The disease is a congenital defect of histidine-ammonia-lyase, an enzyme that belongs to the lyase class (EC 4.3.1.3, gene HAL, 12q22-q23) and catalyzes the deamination of L-histidine to form urocanic acid and ammonia. Histidine (L-α-amino-β-imidazolylpropionic acid) is an essential amino acid which must be present in the diet of young children. Besides, a histidine residue is often present in the active centers of enzyme molecules, and the biosynthesis of histamine in the human organism requires histidine as well. The metabolic block leads to accumulation of large amounts of histidine and the products of its abnormal metabolism (imidazolepyruvic, imidazolelactic, and imidazoleacetic acids) in the tissues and body fluids of the patient, this having a toxic effect on the central nervous system [8, 9, 14, 19, 24].

Diet therapy based on limiting the ingestion of histidine to a value that is the most appropriate for the individual metabolic requirements of the patient’s organism is the only efficient treatment for histidinemia currently available [6, 7, 13, 20].

The aim of the present work was to study histidine biotransformation in milk protein hydrolysates treated by L-histidine-ammonia-lyase, to develop a technique for biotransformation, and to elaborate a procedure for the production of a milk protein concentrate for use in specialized dietary products.

OBJECTS AND METHODS OF THE STUDY

Casein hydrolyzate obtained by treating milk proteins with an enzyme system including exo- and endopeptidases thermolysin, carboxypeptidase A, and leucine aminopeptidase for 8 ± 0.05 h at a temperature of 50 ± 1°C and the enzyme-substrate ratio of 1:50 was used in the present work. Other materials included L-histidine-ammonia-lyase (8.6 U/mg protein) from Sigma, trisodium citrate 5.5-hydrate approved for food use according to GOST (State Standard) 31227-2004, portable water conforming to GOST (State Standard) 2874, and other chemicals manufactured in Russia and abroad, of a purity not lower than “chemically pure” grade.
Sampling and preparation of the samples for analysis were carried out in accordance with the GOST (State Standard) 26809 “Milk and dairy products. Acceptance procedures, sampling methods, and preparation of samples for analysis”. Histidine biotransformation was carried out using a static procedure in a thermostat (temperature 30 ± 2°C) equipped with a stirrer at pH 9 ± 0.01 (phosphate buffer). These values of pH and temperature are optimal for L-histidine ammonia-lyase used in the present work according to earlier reports [8, 16] and information provided by the manufacturer. The duration of biotransformation ranged from 2 to 8 hours; the ratio of enzyme concentration to the concentration of the protein substrate was 1:25, 1:50, or 1:100. The process was terminated by heat inactivation of the enzyme in the hydrolyzate (90–95°C for 5–10 min).

Concentrations of ammonia and urocanic acid were determined using a KAPEL 105 capillary electrophoresis system according to a procedure developed in the scientific and educational center of Kemerovo Institute of Food Science and Technology.

A rotary evaporator IR-1LT intended for rapid removal of solvents from solutions or suspensions of organic and inorganic compounds by film evaporation under normal or reduced pressure and controlled temperature [10] was used to eliminate ammonia from the reaction mixture.

RESULTS AND DISCUSSION

The kinetics of histidine biotransformation in protein hydrolyzates and the effects of various physical and chemical factors on the process rate have been investigated in the present study.

The dependence of the rate of the biotransformation reaction on histidine concentration is shown in Fig. 1.

Fig. 1. The dependence of the reaction rate on histidine concentration.

Analysis of the data shown in Fig. 1 allowed for the conclusion that the dependence of the rate of the biotransformation reaction on substrate concentration corresponds to a classical hyperbolic curve. The character of the dependence was investigated at substrate concentrations ranging from 300 to 9000 mM. As shown in the graph, the reaction rate is proportional to the substrate concentration at low substrate concentrations. However, the maximal value of the reaction rate is attained at a histidine concentration of 5000 mM. The enzyme is obviously saturated by the substrate at this concentration, and therefore the concentration of the enzyme-substrate complex ES becomes equal to the enzyme concentration. The hydrolysis rate remains constant at higher substrate concentrations, this apparently being due to substrate inhibition.

The data obtained were analyzed using the Lineweaver-Burke method for the linearization of Michaelis-Menten equation. The results are shown in Fig. 2. The method of least squares was used to determine the tangent of the inclination angle numerically equal to $1/V_m$. The coordinate of the intersection of the extrapolated line with the y-axis corresponds to the $K_M/V_m$ value, and that of the point of intersection with the x-axis corresponds to the $K_M$ value.

Fig. 2. Graphical determination of the maximal rate of the enzymatic reaction and the Michaelis constant using the Lineweaver-Burke method.

The value of the Michaelis constant determined in the present study equaled 1745 µM, and the maximal rate of the biotransformation process was 1.25 µmol/min. Similar values ($K_M = 1751$ µM, $V_m = 1.15$) were reported by other researchers who analyzed the effects of L-histidine-ammonia-lyase under model conditions. It is known that $K_M$ may vary to a certain extent, depending on the ratio of histidine isoforms, buffer composition, and the presence of substances regulating enzyme activity in the reaction medium [11, 15].

The accumulation of the reaction products in the course of histidine biotransformation by L-histidine-ammonia-lyase at the previously determined reaction parameters ($K_M = 1751$ µM, $V_m = 1.15$) was analyzed in the subsequent experiments. The optimal conditions for the functioning of L-histidine ammonia-lyase are a temperature of 30 ± 1°C and pH 8.5 [15, 22, 23]. Therefore, the dynamics of ammonia and urocanic acid accumulation have been analyzed for a reaction carried out in tetraborate buffer, pH = 9.2 ($\lambda = 254$ nm, voltage 25 kV). The results of the experiment are presented in Fig. 3.
Analysis of the data presented in Fig. 3 allows for the conclusion that the biotransformation results in intense accumulation of ammonia and urocanic acid, indicative of histidine conversion into the reaction products mediated by L-histidine-ammonia-lyase [18]. Thus, the content of histidine dropped to 71.3 % of the initial value after 2 ± 0.05 h of treatment, to 32.06 % of the initial value after 4 ± 0.05 h, and reached the minimal value at 8 ± 0.05 h. Amino acid composition of the hydrolyzates obtained is shown in Table 1.

In the experiments described above, the action of highly specific L-histidine-ammonia-lyase resulted in the formation of metabolites, namely, ammonia and urocanic acid. Ammonia is the terminal product of nitrogen metabolism in humans and animals; nevertheless, it is highly toxic. Therefore, ammonia concentration should be maintained at a low level (the normal level is below 60 µM), and consequently, a technology for the removal of biotransformation products from the hydrolyzates obtained has been developed.

Removal of the by-products of histidine biotransformation (ammonia and urocanic acid) from the casein hydrolyzates resulted in the development of a technique compliant with the requirements concerning histidine-free foods or foods with a low content of histidine [8]. A two-stage process was used for hydrolyzate purification, with ammonia removal performed at the first stage. According to published data, ammonia is a colorless gas with a characteristic pungent smell, readily soluble in water, and with a density almost twice lower than that of the air. As the temperature increases, the solubility of ammonia decreases and evaporation occurs. Therefore, an attempt to delineate the optimal parameters of ammonia elimination by varying the temperature and the duration of the process at a pressure of 0.75 MPa has been undertaken. The results of the experiments are shown in Fig. 4.

![Fig. 3. Dynamics of ammonia and urocanic acid accumulation during histidine biotransformation mediated by L-histidine-ammonia-lyase, after: a) 2 ± 0.05 h, b) 4 ± 0.05 h, c) 8 ± 0.05 h; 1 – histidine; 2 – ammonia, 3 – urocanic acid.](image)

![Fig. 4. Dynamics of ammonia elimination during the purification process: 1-at 60 ± 5°C; 2-at 70 ± 5°C; 3-at 80 ± 5°C.](image)
The data obtained show that the content of ammonia is reduced by 76.5% at 60 ± 5°C and by 89.5% at 70 ± 5°C; this does not provide for the removal of ammonia to a sufficiently high extent, and therefore the purification parameters named above cannot be used in a procedure for the production of a milk protein substitute for specialized functional foods conforming to the standards currently in force [8]. However, ammonia is completely eliminated from the reaction mixture after 90 minutes of treatment at 80 ± 5°C.

Removal of urocanic acid from the hydrolyzates obtained constituted the second step of the procedure. Urocanic acid is removed from solutions by precipitation at low temperature. Therefore, subsequent experiments were aimed at selecting conditions (temperature and duration of treatment) for the most efficient removal of urocanic acid.

The results of the experiments are presented in Fig. 5.

**Fig. 5.** Dynamics of urocanic acid removal during the purification process: 1— at a temperature of 60 ± 5°C, 2— at 70 ± 5°C, 3— at 80 ± 5°C.

Therefore, one can conclude that the temperature of 80 ± 5°C and the process duration of 90 ± 1 min are the optimal conditions for the removal of urocanic acid. The absorption spectra of ammonia and urocanic acid before and after purification are shown in Fig. 6.

**Fig. 6.** Absorption spectra: 1— ammonia, 2— urocanic acid; a) before purification, b) after purification.

The content of ammonia and urocanic acid before and after purification are shown in Table 2.

**Table 2.** The content of ammonia and urocanic acid before and after purification

| Name          | Content, mg/100 g |
|---------------|-------------------|
|               | Before purification | After purification |
| Ammonia       | 3.27±0.05         | 0.001±0.0001       |
| Urocanic acid | 5.64±0.03         | 0.001±0.0001       |

The content of the biotransformation products in the hydrolyzates obtained is shown in Table 2.

The data presented in Table 2 show that the parameters selected for the treatment of the hydrolyzates obtained allow for complete removal of histidine biotransformation products from the reaction mixture and therefore the hydrolyzates obtained can be used as a milk protein equivalent in the manufacturing of specialized dietary products for histidinemia patients.

Further research will be focused on the development of a technology for the production of a milk protein equivalent for use in specialized dietary products for the nutrition of histidinemia patients.

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