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Article

Whey Protein, Soy Protein Isolate Hydrolysate, and Amino Acid Mixture in Adaptation to Physical Stress of Rats

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Abstract: Peptides of hydrolysates of food proteins are an easily digestible source of amino acids necessary for the body to adapt to physical stress. Commercially significant hydrolysates include whey protein, casein, and other animal proteins. Hydrolysates of plant proteins are gaining popularity, but they are less common, then animal ones. Soy protein isolate is promising for obtaining the hydrolysates due to its affordable price and balanced amino acid profile. However, there are no direct studies showing an improvement in the result of physical activity when eating soy protein isolate hydrolysate (SPIH). In this work, for the first time, the study was conducted on the safety and efficacy of SPIH during physical load on model animals (rats). It was shown that the hydrolysate did not lead to pathological changes in the viscera, food intake, and weight of animals did not differ from the control group (animals consumed whey protein). Under physical load rats enteral fed SPIH showed a tendency to adapt more quickly to physical stress than the control group and the group of animals that was fed by free amino acids. The metabolites of animal blood serum were studied by NMR spectroscopy. It was shown that by the 95th minute after feeding in the group of rats receiving SPIH, the difference of proteinogenic amino acids concentrations in blood between individuals was significantly less than in the groups receiving whey protein or a mixture of amino acids. In other words, individual biochemical and physiological characteristics of individuals did not affect the assimilation of amino acids of hydrolysate.

Keywords: soy protein isolate; protein hydrolysate; soy protein peptides; physical load; food efficacy and safety; NMR blood test; blood metabolites; forced swimming

1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), animal husbandry makes a significant contribution to the pollution of water resources, mainly in the process of growing fodder crops for livestock. 55% of soil erosion and deposition, 37% of the use of pesticides, 50% of the use of antibiotics, and a third of the pollution of water resources with nitrogen and phosphorus are associated with animal husbandry [1]. The trend of switching from animal to vegetable food is mainly determined by the listed environmental problems, as well as the increased demand for vegetable food in the market due to the development of the culture of vegetarianism and veganism.

Soybean is the main source of protein used in human nutrition [2,3]. Soy protein is similar to the "ideal protein" proposed by the joint report of the World Health Organization, the Food and Drug Administration (FDA), and the FAO. In addition, the percentage of protein in soybean is 40–50%, which makes soy an economically viable raw material for the production of protein concentrates and isolates. Concentrates are obtained by removing soluble sugars from low-fat soy flour (US3971856A). Isolates are obtained by dissolving soy

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protein, cleaning the solution from flakes and subsequent precipitation of protein at pH 4–5 (US3397991A).

Protein concentrates and isolates are convenient raw materials for obtaining an easily digestible form of protein — hydrolysates. Hydrolysates contain peptides and free amino acids and are an ingredient of a sports and functional food product.

Reviews of studies of the effect of food proteins hydrolysates on the biochemical and physiological parameters of the body were published earlier [4–6]. One of the first studies showing the physiological effect and high degree of bioavailability of animal proteins hydrolysates were carried out on starving rats [7,8]. Rats that were fed by hydrolysate gained weight faster, nitrogen was absorbed more efficiently. The study of the effect of consumption of whey protein hydrolysate before sprint interval training was carried out on humans. The blood serum metabolites, the expression of certain genes and the level of acetylation of muscle tissue proteins were studied, and the performance of physical exercises (aerobic and anaerobic performance) was evaluated [9]. The concentration of threonine, glycine and ornithine in the blood serum in the group that consumed protein hydrolysate was higher relative to the group that received native protein.

A study of the kinetics of the accumulation of free amino acids in the blood after eating plant protein was conducted on people without physical activity [10]. It was shown that amino acids of glycine hydrolysate (the main soy protein) were absorbed more efficiently than amino acids of native protein or mixtures of free amino acids (the same amino acid profile). Morifuji et al. compared the rate of occurrence of free amino acids, dipeptides, and the insulin concentration in the blood in the subjects who took whey or soy protein in native or hydrolyzed form at rest [11]. The intake of protein hydrolysates provided a faster appearance of amino acids in the blood, but in the case of whey protein hydrolysate, the increase in the concentration of essential and BCAA amino acids occurred much faster than when taking soy protein isolate hydrolysate (SPIH). In this work, it was shown that hydrolysates (without carbohydrates in experimental food) increased the concentration of insulin in the blood faster than non-hydrolyzed protein. Presumably, after ingestion of hydrolysates together with carbohydrates, an increase in insulin in the blood can lead to rapid saturation of cells with glucose and thereby contribute to the rapid adaptation of the body to physical load [5,6]. Until now, studies of the effect of SPIH on the effectiveness of physical load have not been conducted.

Protein hydrolysates are also sources of biologically active peptides (BAP). BAP are short chains of amino acids (from two to several dozens in a chain) that have the ability to bind to enzymes or receptors in functional parts and thereby influence their properties. BAP is often found in the products of enzymatic hydrolysis of food proteins [12]. According to the Pubmed database, the use of the words "bioactive peptides food" in the title and abstract, since 1982, is found in 2,073 scientific publications (June 23, 2022). The growth of such publications is exponential. For example, dozens of peptides of soy proteins have been identified that exhibit antihypertensive, anti-cancer and many other properties [13]. Therefore, hydrolysates of food proteins should be considered not only as a source of amino acids, but also as a source of BAP that can affect the physiological parameters of the body that are not directly related to the synthesis of muscle and other proteins. These changes in physiology might have a significant impact on the result of physical activity both in a positive and negative way.

In this work, the effect of SPIH on the rate of adaptation to physical stress in model animals was studied for the first time. On the 7th day of physical load a statistically significant increase in the swimming time in the test after preliminary enteral administration of both soy protein hydrolysate and whey protein was shown. A group of animals receiving a mixture of amino acids showed a statistically significant difference between the swimming time of the first and 11th day of physical load. No pathological changes in the weight of viscera were detected in all three groups, which indicates the safety of consumption of SPIH as food. Analysis of serum metabolites using NMR spectroscopy showed that the group of rats consuming SPIH was more homogeneous in terms of metabolite concentrations, unlike

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the groups consuming native whey protein or a mixture of free amino acids. That is, the assimilation of SPIH does not depend on the individual characteristics of individuals.

2. Materials and Methods

2.1. Preparation of a protein-carbohydrate mixture

To obtain a protein-carbohydrate mixture based on whey protein, 1.681 kg of deionized water was added to the homogenizer "Shredder mixer IS-5" (Limited Company small-scole innovation Enterprise "BioPischeMash", Russia), and heated to 50 °C, after which it was added 694 g of Lactomin whey protein concentrate (Fonterra). The concentrate manufacturer claims that the product contains 80% of protein. However, our examination of the amino acid profile showed that it contains 73% of protein. Therefore, further calculations during the preparation of the protein-carbohydrate mixture were made on the basis of a protein content of 73%. Then the mixture was being stirred at 50 °C 10 min at 1,100 rpm of the rotor-stator, and then 20 min at 550 rpm. At the end of mixing, 125 g of edible sugar (99%) was added, mixed at 50 °C for 10 min at 550 rpm and cooled to 25 °C. The resulting mixture was poured 30–40 ml into sterile falcon-type test tubes, which were stored at −30 °C.

To prepare an amino acid-carbohydrate mixture, 2 kg of deionized water was added to IS-5 and heated to 50 °C, after which 564 g of food additive grade amino acids premix was added (the purity of each amino acid preparation was evaluated using NMR spectroscopy (Tab. A1)). The amino acid profile of this premix corresponded to the profile of the used SPI. The amino acid profile of the SPI was determined in the analytical laboratory "Test-Pushchino" (Russia) on the amino acid analyzer SYKAM S430 ("Sykam", Germany) after the acid hydrolysis procedure. Since after acid hydrolysis asparagine is converted into aspartic acid, and glutamine into glutamic acid, the exact amount of each of these amino acids is not known. In this regard, the ratio of asparagine to aspartic acid and glutamine to glutamic acid as 1 to 1 was used to prepare the premix. Amino acid solution stirred at 50 °C 10 min at 1,100 rpm of the rotor-stator, and then 20 min at 550 rpm. At the end of mixing, 135 g of edible sugar (99%) was added to the reaction mixture, then the mixture was mixed at 50 °C for 10 min at 550 rpm of the rotor-stator and poured 40 ml into sterile falcon-type test tubes, which were stored at −30 °C.

To obtain a protein-carbohydrate mixture based on soy protein isolate hydrolysate (SPIH), 2 kg of deionized water was added to IS-5, heated to 50 °C, then 610 g of SPI Shansong 90 (Linyi Shansong Biological Products Co., Ltd., China) and 3.7 ml of the enzyme preparation Alkalase 2.4 L FG (Novozymes, Denmark). The activity of the Alkalase preparation was 2,933 units/ml (measured as in [14]). The number of activity units in the reaction mixture was 17.8 units/1 g of SPI). The mixture was stirred for 2 h at 50 °C at a speed of 1,100 rpm. To inactivate the enzyme, the reaction mixture was heated to 67 °C. The mixture was kept for 10 min and then cooled to 22 °C. After cooling, 135.85 g of edible sugar (99%) was added. The mixture was stirred at room temperature at 550 rpm for 10 min. The mixture was subdivided into individual 80 falcon-type tubes of 35 ml each, which were stored at −30°C. The concentrations of protein and sugar in each mixture are indicated in Tab. 1.

Table 1. The composition of the protein-carbohydrate mixture

The source of amino acids	Amount of protein/amino acids, %	Amount of sugar, %
Whey Protein	20.26	5.00
SPI hydrolysate	19.99	4.95
A mixture of amino acids	20.90	5,00

Sterilization of protein-carbohydrate mixtures was carried out using gamma irradiation at Sterus-1 (MRTI) with the following parameters. Absorbed dose of ionizing radiation was 15 KGF, current was 840 μA, pulse frequency was 150 Hz.

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2.2. Experiment design

2.2.1. Animals

Sexually mature male rats of the outbred Wistar stock were used. At the time of randomization, the rats were 12 weeks old, 280.8 g., SD=21.4 g. This animal model is recognized as suitable for studying nutrition problems and adaptation to physical stress (swimming) [15–17]. The initial sample size was 10 individuals in each group. The animals were obtained from the The Lab Animals Breeding Center, Branch of Institute of Bioorganic Chemistry RAS (www.spf-animals.ru). They were quarantined for 14 days and then kept in standard vivarium conditions at a temperature of 21–23 °C, relative humidity 40–60%, light mode 12:12 turning on the light at 08:00. The animals were fed balanced granulated fodder ad libitum and had free access to drinking water. The animals were kept in 6 individuals in Eurostandart Type IV S plastic cages ("Techniplast", FarmBioLine, Russia) with metal grilles and metal dividers for feed and for drinking water.

2.2.2. Randomization

The study was conducted according to protocols approved by the Ethics Commission of the Institute of Theoretical and Experimental Biophysics of the Russian Academy of Sciences. The work was carried out in compliance with the principles of the Helsinki Declaration on Humane Treatment of Animals and the principles of humanity set out in the Directive of the European Community (86/609/ECC), in the State Standard 33215-2014 dated 07.01.2016 "Guidelines for the maintenance and care of laboratory animals. Rules of equipping the premises and organization of procedures". After the quarantine/adaptation period the animals which did not have health abnormalities were selected. Randomization of animals was carried out according to two parameters: body weight and swimming time to exhaustion (under conditions similar to experimental ones, but without enteral feeding). The average values of body weight and swimming time in animals did not differ statistically between the groups. When distributing into groups, the principle of random selection was respected.

2.2.3. Physical load test after enteral feeding

Food deprivation was carried out 4 h before the start of the every test day. The test was carried out in a smooth plastic cylindrical container with a diameter of 40 cm and a height of 100 cm. The height of the water column was 60 cm, the water temperature was 25±1 °C. The change of water in the tank was carried out after swimming 3 animals. The duration of the swim was recorded using a stopwatch with an accuracy of 1 second. The animals were weighed before each swim with an accuracy of 1 g. Individual body weight data were used to calculate the weight of the load, which was 5% of the body weight. Before swimming, the animal was put on a vest made of waterproof fabric with a weighting. The animals were tested during the period 12:00-15:00 h (Moscow time) 3 times a week (every other day) for 4 weeks. The physical endurance of animals in the test "Forced swimming with a load to exhaustion" was evaluated by the duration of swimming after preliminary (30 min before the test) enteral administration of a protein-carbohydrate mixture based on whey protein, or hydrolysate of soy protein isolate, or a mixture of amino acids (10 g of the mixture per 1 kg of the animal's body weight). In the same time interval there was one animal in a container with water. The animal swam to exhaustion. The moment of the end of the experiment was considered the fatigue of the animal, manifested in the refusal or impossibility of swimming (with passive immersion to the bottom for 10 sec). The results of the first swim after enteral feeding were taken as 100% when analyzing relative increase in swimming time.

2.2.4. Blood sampling and necroscopy of animals

With planned necropsy (as part of euthanasia) on the 39th and 40th days of the experiment, blood was taken terminally after 95 min of enteral administration. Anesthesia was performed with Telazol®/Xyla®. Terminal blood collection was taken from the inferior vena cava during laparotomy, using a syringe with a needle with a diameter of 26G.

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A complete necropsy of the animals was performed. Necropsy included examination of the external surface of the body, all openings, cranial cavity, external surface of the brain, thoracic, abdominal and pelvic cavities, including viscera. The following organs were taken and weighed: liver, kidneys, adrenal glands, heart, lungs, testes, spleen.

2.3. Statistical analysis

The sample size on a particular experimental day varied for several reasons. Animal mortality was 4 rats per 30. During enteral administration, one individual was injured in the esophagus and was euthanized. As the autopsy showed, the second rat dropped out due to a stroke while swimming. The other two dropped out for unknown reasons (the bodies were found 10 h after the estimated time of death — conducting an autopsy was useless). No significant deviations in the behavior or body weight of these two individuals were observed. The data obtained before being eliminated from the experiment were taken into account in statistical processing. The data obtained from the rats who immediately refused to swim, or who managed to take off their vest while swimming, were not taken into account in the statistical processing of this experimental day. The size for statistical analysis for each presented experimental result is specified in detail in Application Tab. A3.

Statistically significant difference using nonparametric paired Wilcoxon statistics was detected in the Statistica program. A pair of samples of the D0 type was formed. D0 is a set of swimming times within this group (WP, SPIH or AAM) on the zero day of physical load and Dn is a set of swimming times within this group on the N day of swimming, after which the significance of the differences between these samples was evaluated. The null hypothesis was the hypothesis about the equality of the medians of these samples.

2.4. NMR spectroscopy of methanol soluble metabolites of blood serum

Samples for NMR spectroscopy were carried out on the basis of previously developed protocols for the study of serum metabolites [18,19] with minor changes (Fig. 1). Blood was incubated at 37 °C for 2 h, then 3 volumes of saline solution containing 0.12% sodium azide and 0.2 mM trimethylsilyl sodium propionate (3-(Trimethylsilyl)propionic-2,2,3,3-d 4 acid, sodium salt; TSP) were added. The mixture was gently stirred and centrifuged at 822 g and 4 °C for 10 min. $\geq$2 volumes of methanol cooled at −20 °C were added to the filler liquid, stirred and incubated for 30 min at −20 °C. Further, centrifugation was carried out at 12,100 g, 10 min, at 4 °C. The infusion fluid was incubated overnight at −40 °C and lyophilized. Dried samples were stored at −20 °C in hermetically sealed mini-samples. Immediately before the NMR analysis, these samples were dissolved in a phosphate buffer (100 mM, pH 7) and heavy water up to 5% of its final concentration. One-dimensional (1D) ^{1}H-NMR spectra were acquired with a Bruker Avance III 600 spectrometer (The Core Facilities Centre of Institute of Theoretical and Experimental Biophysics of the RAS) operating at a frequency of 600 MHz (1H).

3. Results

3.1. The effectiveness of physical load

Test "Forced swimming with a load to exhaustion" has its advantages in studies on adaptation to physical stress over other types of physical activity, for example running [16]. Rats are good swimmers, swimming is not traumatic, the moment of exhaustion is easily detected visually. The disadvantage of the test is the high variability in swimming time both within groups and between the results of works published earlier, where this test was used (for an comprehensive review of such works see [16]).

In this study 30 min before the test, three groups of rats were enterally administered with a protein-carbohydrate mixture based on whey protein (WP group), soy protein isolate hydrolysate (SPIH group), or a mixture of amino acids (AAM group; the amino acid profile corresponded to soy protein isolate (SPI), Application Fig. A2). A mixture based on native SPI in a concentration of 20% protein formed a dough-like mass, which was not possible to manipulate during the enteral feeding procedure. Therefore, whey protein concentrate was

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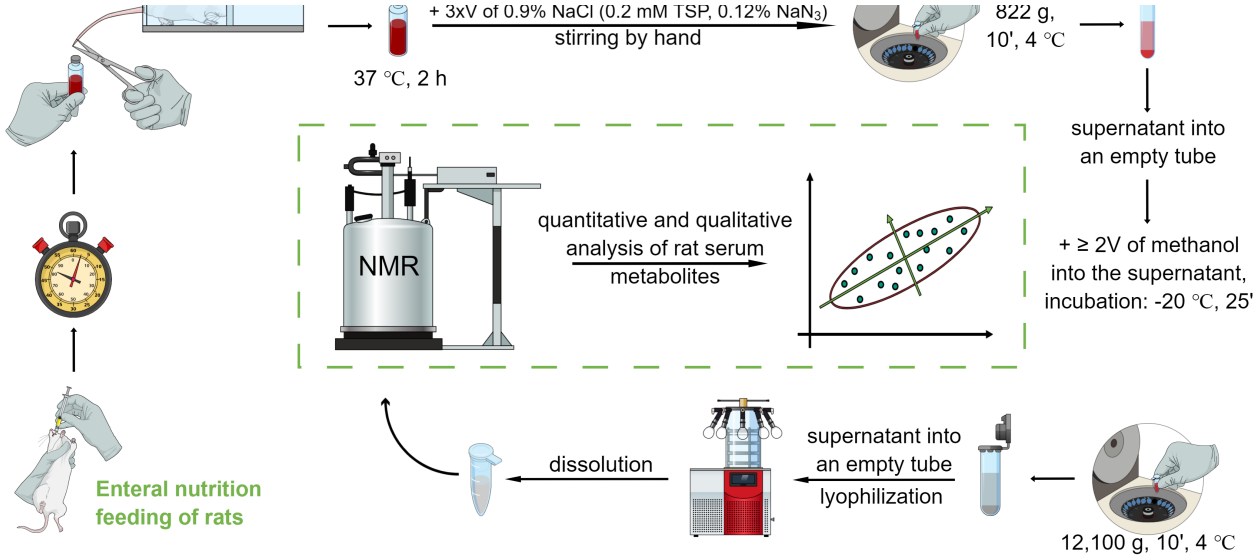

Figure 1. Scheme of preparation of blood samples for NMR studies

used as a control of the native protein. Prior to the start of the experiment, randomization of animals was carried out by weight and by the test swimming time (without enteral administration of the mixture). Throughout the experiment, before the test, the animals were weighed. The feed was previously withdrawn 4 h before. Water was freely available. 30 min before the start of the test, the animals were enterally administered with mixtures (10 g of mixture per 1 kg of animal body weight). The first day of physical load with enteral feeding was taken for the "zero day". For the result of swimming in 100%, a "zero day" of physical load with enteral feeding was taken. The results of the physical test are given at Fig. 2. Individuals of the SPIH group showed a progressive tendency to increase swimming time, starting from the 4th day of physical load. On the 11th day of the test, the SPIH group showed the increase in swimming time on average 500%. The WP and AAM groups did not show such a clear trend. In absolute values, the SPIH group increased its swimming time by an average of 300 seconds during the entire experiment (Application Fig. A1).

Statistically significant difference was detected using nonparametric paired Wilcoxon statistics. The WP and SPIH groups show a statistically significant increase in swimming time on the 7th day of physical activity with enteral feeding (Fig. 3). The group receiving a mixture of amino acids showed a statistically significant increase in swimming time only on the last day of the experiment.

3.2. Animal body weight gain

The body weight of the animals in all three groups increased throughout the experiment (Application Fig. A2). The average body weight gain does not depend on the type of enteral feeding mixture, (Application Fig. A2(a)–(c)), however, the nature of the change in the standard deviation (SD) was significantly different (Application Fig. A2(d)). In addition to the 7th day of the test, in the SPIH group, the SD value remained at the same level, while in the WP and AAM groups, the SD value increased throughout the experiment. By the end of the experiment the SD value increased several times compared to the first days of the test in these two groups. Feed intake during the experiment did not differ between the three groups (Tab. A4).

3.3. Weight of viscera of animals

Significant differences in the weight of viscera (relative to the weight of the animal) (adrenal glands, liver, testes, kidneys, thymus, spleen, heart) between the three groups

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were not recorded (Application Fig. A3). The weight of the organs of adult male Wistar rats corresponds to the norm of male Wistar rats of this age [20]. There were no external pathological changes.

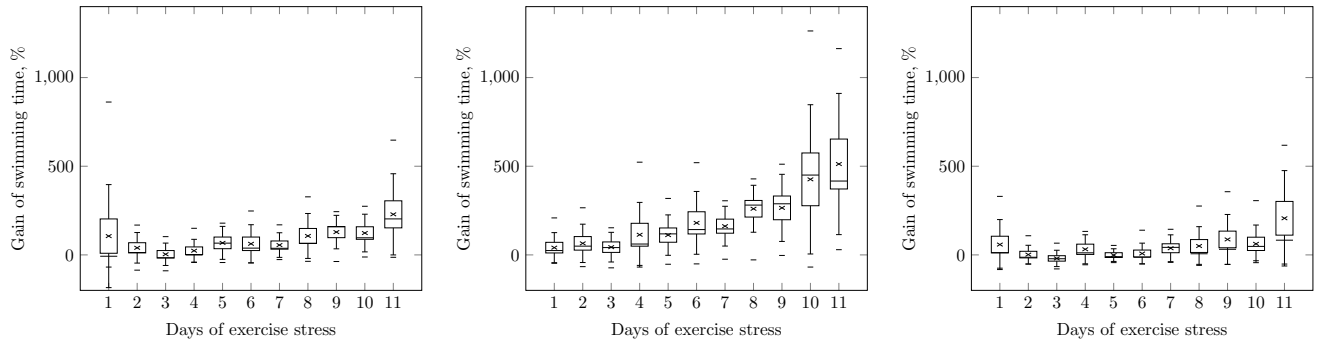

Figure 2. Relative increase in swimming time to exhaustion in the group of animals receiving enterally whey protein (a), soy protein isolate hydrolysate (b), amino acid mixture (c) 30 min before the start of the "Forced swimming with load until exhaustion". The following characteristics of the sample are graphically presented: the minimum and maximum of the sample with separate horizontal lines, the average — with a diagonal cross, a box — the interval ±SEM (standard error of the mean), a horizontal long line — the median, the whiskers — one standard deviation (±SD). For the sample size, see Application Tab. A3.

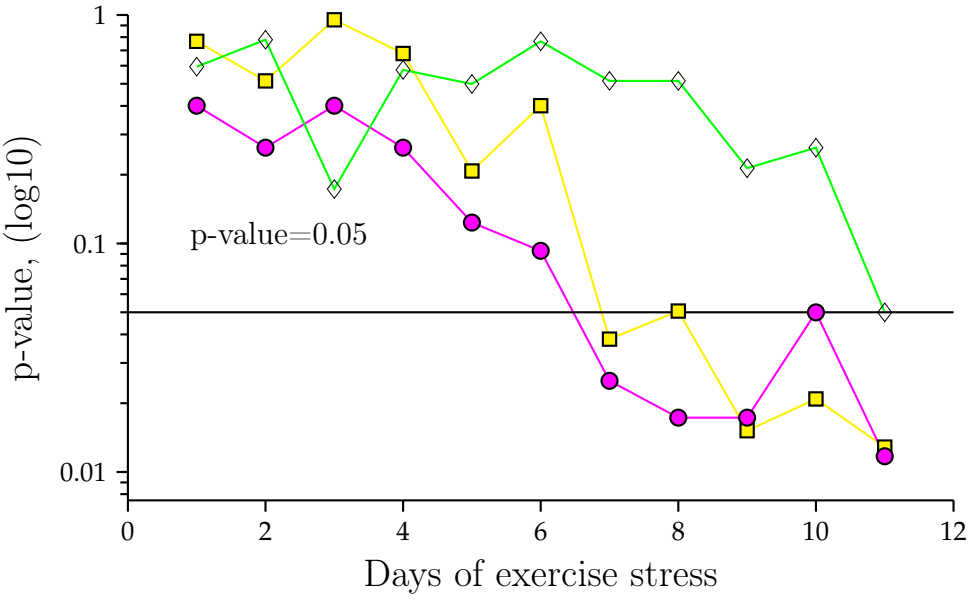

Figure 3. p-values, obtained when applying the 1-tailed Wilcoxon paired non-parametric test (Wilcoxon signed-rank test) on the Statistica software comparing the swimming time of each group on each loading day with the swimming time of the same group on the zero loading day: — group WP, — group SPIH, — group AAM. Sample size see Application Tab. A3

3.4. Blood serum metabolites

To conduct a quantitative analysis of serum metabolites, blood proteins were removed by methanol precipitation. The supernatant was dried, after which an NMR analysis was performed. Using NMR spectroscopy, 53 serum metabolites (Application Tab. A5) were identified and quantified, 19 of which are proteinogenic amino acids (the spectrum of cysteine overlaps with the spectra of other substances, and therefore its quantitative determination is impossible). The principal component analysis (PCA) showed that by the 95th minute after enteral administration of protein-carbohydrate mixtures (without physical load on the day of blood collection), serum metabolites of three groups of animals formed three overlapping clusters (Fig. 4). When analyzing of 34 metabolites — without proteinogenic

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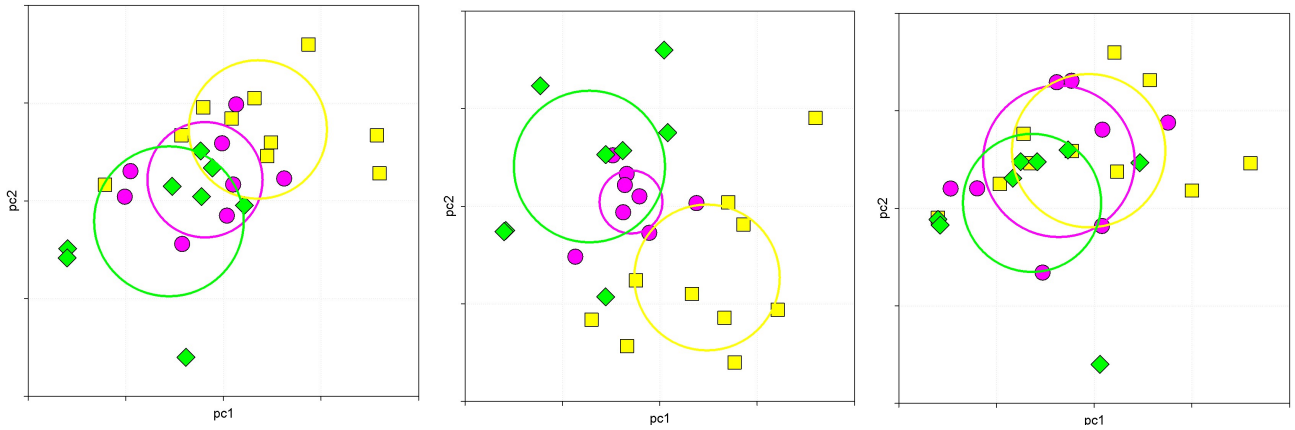
amino acids — the significant overlap was observed. If the data obtained from each group is described by a circular distribution, where the average value of the coordinates in PCA is taken as the center of the circle, and the average value of the distances from the center to each point in each group is taken as the radius, then we can estimate the relative degree of compactness of each group through the ratio of areas (Fig. 4d). The relative areas in the PCA of the WP and AAM animal groups exceeded the area of the SPIH group by one and a half times when analyzing all 53 metabolites (Fig. 4a) and especially when analyzing only amino acids (Fig. 4b). In the PCA of metabolites except proteinogenic amino acids, the circular distribution areas were approximately the same. Thus, the SPIH group in the PCA method turned out to be more compact in terms of amino acid concentrations in the blood. That is, the values of amino acid concentrations in the blood of the SPIH group of rats had a relatively smaller dispersion than in the WP and AAM groups.



(a) (b) (c)

In the PCA	The relative area of the circular distribution		
	WP	SPIH	AAM
All metabolites (a)	1.47	1	1.71
Amino acids only (b)	5.39	1	5.78
All except amino acids (c)	1.02	1	0.84

(d)

Figure 4. Principal component analysis (PCA) of the concentrations of serum metabolites of rats receiving enterally whey protein (—□—), soy protein isolate hydrolysate (—○—), or a mixture of amino acids (—◇—): (a) 53 metabolites, (b) 19 protein amino acids, (c) 34 metabolites (without proteinogenic amino acids), (d) the ratio of the circular distribution areas describing the spread of the concentration of serum metabolites of animal groups in the principal component analysis.

4. Discussion

One of the reasons for the low digestibility of amino acids of plant proteins is the content of anti-nutritional factors, for example, inhibitors of proteolytic and other digestive enzymes [21]. The quantitative content of these anti-nutritional factors is significantly reduced due to modern technologies for processing plant raw materials into protein concentrates and isolates. Enzymatic proteolysis is one of the ways to inactivate some inhibitors of a protein nature. For example, soy Kunitz-type trypsin inhibitor is cleaved by proteolytic enzymes of the culture fluid of bacteria of the genus Bacillus [22], which is often the basis of commercial drugs approved for use in the food industry (for example, the Alkalase used in this work). That is, enzymatic hydrolysis not only increases the bioavailability of amino acids due to the peptide form of protein in hydrolysates, but also can reduce the activity of some anti-nutritional factors of plant protein foods. The study of the residual activity of anti-nutritional factors of the soy protein isolate hydrolysate (SPIH) used in this work is a separate scientific task.

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Usually, digestibility studies are carried out on hydrolysates with a low molecular weight of peptides (Tab. A6). To do this, a step-by-step hydrolysis is carried out with several enzymes, sometimes with the use of fractionation. In our work, we investigated a hydrolysate, a relatively simple technology for the production of which we had previously worked out on a semi-industrial scale [14]. The technology boils down to the use of one commercially available enzyme preparation without any additional complex procedures, the distribution of molecular weights of the peptides obtained: <1 kDa ∼ 5.5%, 1–12.5 kDa ∼ 72%, >12.5 kDa ∼ 22%. Thus, this hydrolysate is easy to reproduce for further research, and the prevalence of di- and tripeptides is not a mandatory characteristic of a hydrolysate with functional properties.

The hydrolysate studied in this work is prepared on the basis of a commercially available soy protein isolate (SPI) and an Alkalase preparation approved in the food industry without the use of any additional reagents. Despite this, we conducted a safety study on rats and showed no toxic effect of consuming hydrolysate three times a week before phisical load for 32 days. The body weight gain of all three groups of animals continued throughout the experiment, which coincides with the data in the group of Wistar rats of the same age without any manipulation in previously published works [15,20]. The feed intake during the whole experiment did not differ between all three groups. The relative weight of organs in all three study groups also does not significantly differ from the data of control groups of another studies [20]. Normal body weight gain and appetite (feed intake) and the absence of external and weight pathologies of animal organs indicate the safety of consumption of all three enteral feeding mixtures before physical exertion during 12 exercises.

The main expectation from the consumption of protein hydrolysates by athletes is an improvement in the performance of physical activity due to the rapid appearance of amino acids in the blood [4–6,9]. Despite a large number of studies of the effect of hydrolysates on the concentration of amino acids in the blood and less often in muscle tissue and other biochemical parameters, direct evidence that hydrolysates can improve exercise performance is very small (for example, [9]). In this paper, we have shown for the first time that the consumption of SPIH immediately before exercise can really accelerate adaptation to physical stress. We have shown that enteral feeding of animals with whey protein or a mixture of amino acids increased the average swimming time of rats at the end of the experiment by 2 times (Application Fig. A4), while the SPIH group improved their performance by an average of 5 times.

The observed acceleration of adaptation can be caused by several reasons. The simplest explanation is amino acids of hydrolysate peptides are absorbed faster in the gastrointestinal tract and are in sufficient concentrations in the blood at the start of the test. During physical exertion, some of the received amino acids participate in energy production (citric acid cycle; 20 of the 20 amino acids) or glucose synthesis (hepatic gluconeogenesis; 18 of the 20 amino acids excluding leucine and lysine [23]), others go to maintain muscle protein synthesis and thereby inhibit the mechanism of muscle protein catabolism to maintain the minimum concentration of amino acids in the blood. The processes of catabolism should have affected the weight difference between groups of animals. However, all three groups did not lose weight and had no statistically significant difference in the values of weight gain (Application Fig. A2, Fig. A4). The obtained result still does not withdraw from discussion the relative increase in muscle mass of animals in the SPIH group, since the ratio of muscle mass to adipose tissue of animals is unknown to us.

Glucose is the main substrate of energy pathways during intense physical load. The test (swimming to exhaustion) includes an anaerobic phase in which the glucose reserves in the muscles are rapidly depleted. It is known that peptides increase the level of insulin and thereby accelerate the transport of glucose from the blood to the cells of the body [5,6,11]. This is explained by the stimulation of insulin secretion by β cells of the pancreas through the binding of blood amino acids to cell receptors. Since all three tested mixtures contained 20% protein and 5% sugar, the second explanation for the positive effect of the hydrolysate

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may be an increase in the concentration of insulin in the blood and the rapid transport of glucose into animal muscle cells.

Biologically active peptides (BAP) of SPI hydrolysate could also be the cause of accelerated adaptation to physical stress. For example, a decrease in blood pressure in SPIH rats during exercise could probably have had a positive effect on the test result. However, to our knowledge, no direct experiments on the effect of BAP on the body during phisical exertion have been conducted.

The most interesting observation is the result of the analysis of animal blood metabolites using NMR-method and the principal component method (Fig. 4). It turned out that the SPIH group at the end of the experiment and 95 min after enteral feeding was more homogeneous in the concentrations of amino acids (there is a crowding of individuals in one area in the principal component analysis). The WP and AAM groups were more heterogeneous in this indicator (the area describing the sample is relatively high). It can be assumed that the individual biochemical characteristics of animals in the case of the SPIH group had little effect on the assimilation of amino acids of the SPI hydrolysate.

Before the amino acids get into the blood, the protein must be broken down to peptides and free amino acids by several proteolytic enzymes of the gastrointestinal tract. Further, amino acids, di- and tripeptides are transported from the lumen into intestinal enterocytes by special protein transporters, and then either degraded by enterocyte enzymes to amino acids (for peptides), or immediately transported into the blood. In the blood, di- and tripeptides are rapidly cleaved to amino acids by proteolytic enzymes. Thus, many enzymes and transporters are involved in the transport of amino acids into the blood, the genes of which may be in different combinations of alleles in the individuals. According to the obtained results of the analysis of metabolites, it can be assumed that alleles of protein genes that are necessary for the assimilation of whole protein or free amino acids are more variable among animal individuals than genes whose expression is necessary for the assimilation of peptides (for example, the gene of the transporter of di- and tripeptides PepT1).

Regardless of the hypothesis stated above, the consumption of SPIH not only accelerates adaptation to physical stress, but also levels the individual biochemical characteristics of individuals relative to the concentration of blood amino acids. Interestingly, changes in the weight of animals in the SPIH group were also more predictable for a single individual than in the WP and AAM groups (Application Fig. A2(d)).

5. Conclusions

Protein hydrolysates by themselves or as an ingredient of a food product are an already established trend in the food industry. First of all, the demand for such products comes from the sports achievements industry. Also, hydrolysates are vital for people with protein digestion problems.

The main raw materials for the production of hydrolysates are animal proteins. Hydrolysates of plant proteins are much less common, but the modern lifestyle (an increase in supporters of vegetarian and vegan diets), economic, and environmental problems are changing the situation, and in the near future hydrolysates of plant proteins will be just as popular. The hydrolysate of soy protein isolate of the peptide form has every chance to become a leader in consumption in various areas of the food industry. The scientific validity of its safety and effectiveness is confirmed by the results of several studies, including those presented in this article.

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Appendix A.

Table A1. The composition of the premix of free amino acids. The premix contained the amino acid profile of soy protein isolate used in this work.

Amino acid	Purity based on NMR data, %	For the preparation of 100 g of dry premix, mg
Tryptophan (Suzhou Vitajoy Bio-Tech Co.)	99.92	822
Cysteine (cysteine hydrochloride) (Foodchem International Corporation)	76.83	1,021
Methionine (Wuxi Jinghai Amino Acid Co.)	99.89	1,102
Histidine (Dia-m)	99.92	2,110
Threonine (Zhejiang Medecines & Health Production Import & Export Trade Co.)	99.97	3,812
Tyrosine (PanReac AppliChem)	99.80	3,418
Alanine (Suzhou Vitajoy Bio-Tech Co.)	99.90	4,264
Glycine (100ING)	99.95	3,946
Isoleucine (Suzhou Vitajoy Bio-Tech Co.)	98.80	4,317
Valin (WATT-NUTRITION)	99.15	4,341
Proline (100ING)	99.97	5,113
Phenylalanine (Suzhou Vitajoy Bio-Tech Co. Ltd)	99.94	4,873
Serine (Dia-m)	99.20	5,176
Lysine (lysine hydrochloride) (Foodchem International Corporation)	79.89	7,733
Leucine (Xinjiang Fufeng Biotechnologies Co.)	99.00	7,204
Arginine (Zhangjiagang Specom Biochemical Co.)	99.93	7,085
Aspartic Acid (asparagine monohydrate) (Wuxi Jinghai Amino Acid Co.)	99.60	5,848
Asparagine (PanReac AppliChem)	87.12	6,686
Glutamic Acid (monosodium glutamate) (100ING)	86.79	11,303
Glutamine (Foodchem International Corporation)	99.85	9,825

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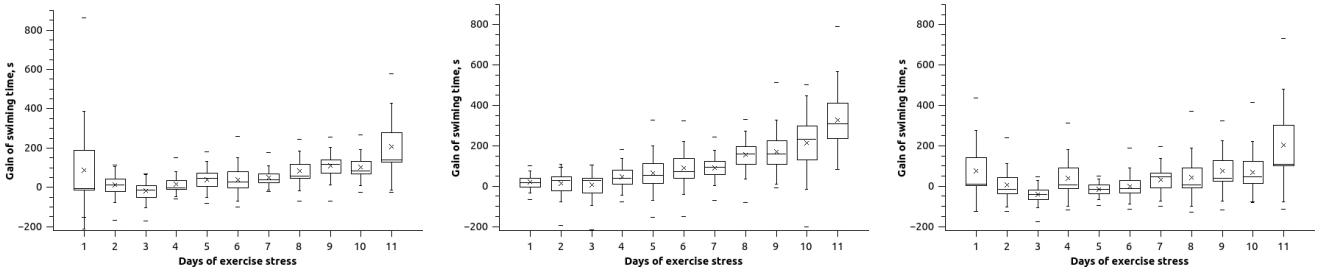
Table A2. Amino acid content in whey protein, soy protein hydrolysate and the mixture of free amino acids used in the experiment

Amino acid	Mixture based on whey protein, mg in 1 g of protein-carbohydrate mixture	Mixture based on SPI hydrolysate, mg in 1 g of protein-carbohydrate mixture	Mixture based on free amino acids, mg in 1 g of amino acid-carbohydrate mixture
Tryptophan	2.97	1.78	1.7
Cysteine	6.94	1.70	1.6
Methionine	4.94	2.38	2.3
Histidine	5.00	4.56	4.4
Threonine	14.16	8.24	8.0
Tyrosine	6.11	7.37	7.1
Alanine	10.83	9.21	8.9
Glycine	3.94	8.53	8.2
Isoleucine	12.77	9.22	8.9
Valine	11.94	9.30	9.0
Proline	13.88	11.05	10.7
Phenylalanine	6.33	10.53	10.2
Serine	9.83	11.10	10.7
Lysine	14.71	13.35	12.9
Leucine	20.54	15.42	14.9
Arginine	4.94	15.30	14.8
Aspartic Acid	10.27	12.59	12.2
Asparagine	10.27	12.59	12.2
Glutamic Acid	16.10	21.20	20.5
Glutamine	16.10	21.20	20.5



(a) (b) (c)

Figure A1. Increase in swimming time to exhaustion in the group of animals receiving enterally whey protein (a), soy protein isolate hydrolysate (b), amino acid mixture (c) 30 min before the start of the test "Forced swimming with a load to exhaustion" . The following characteristics of the sample are graphically presented: the minimum and maximum of the sample with separate horizontal lines, the average — with a diagonal cross, a box — the interval ±SEM (standard error of the mean), a horizontal long line — the median, the whiskers are one standard deviation (±SD). See Application Tab. A3 for sample size.

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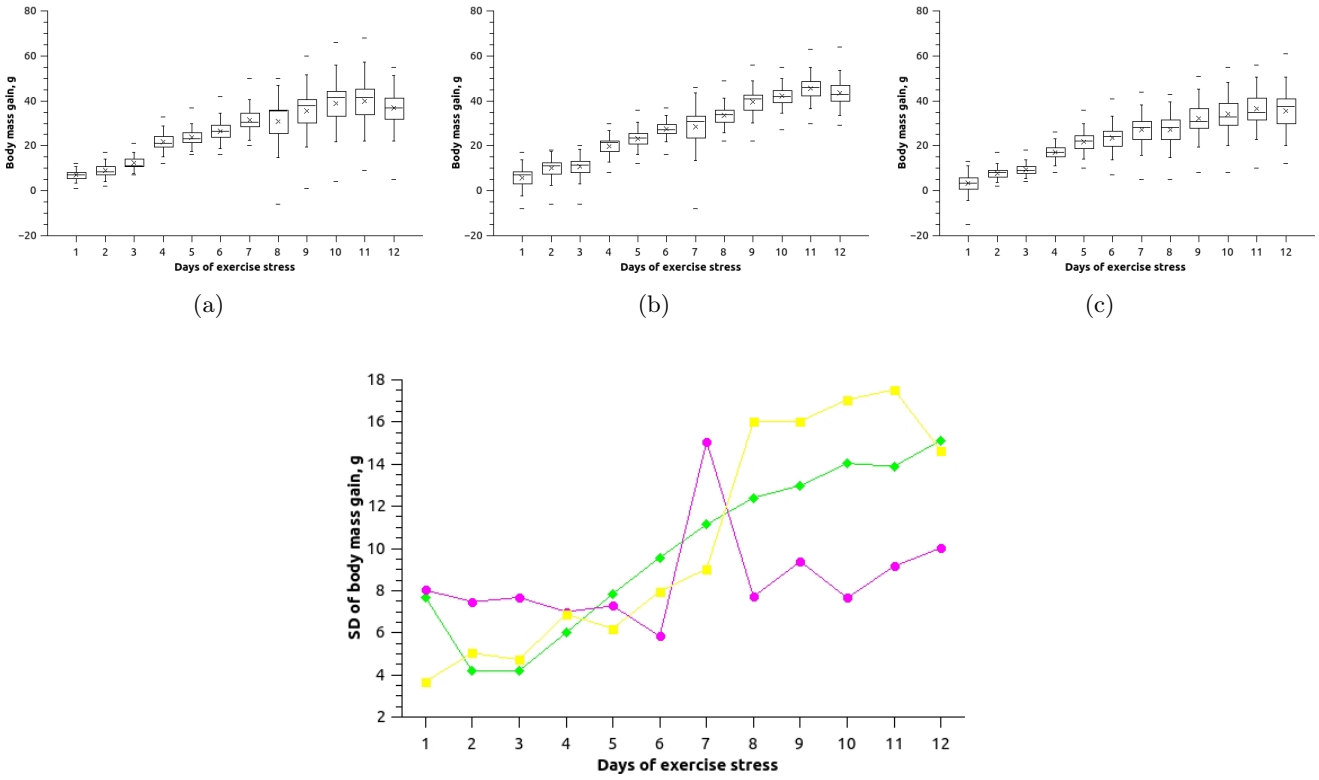

Figure A2. Absolute body weight gain in the group of animals treated enterally with whey protein (a), soy protein isolate hydrolysate (SPIH) (b), amino acid mixture (c) during the experiment. (d) is standard deviation by days in the group of rats treated enterally before physical load (last weighing — 12th day — without enteral feeding and physical load) with whey protein (yellow squares), SPIH (pink circles), or a mixture of amino acids (green diamond). The following characteristics of the sample are graphically presented (a–c): minimum and maximum of the sample with separate horizontal lines, average — diagonal cross, box — interval ±SEM (standard error of the average a horizontal long line — the median, whiskers — standard deviation (±SD). See Application Tab. A3 for sample size.

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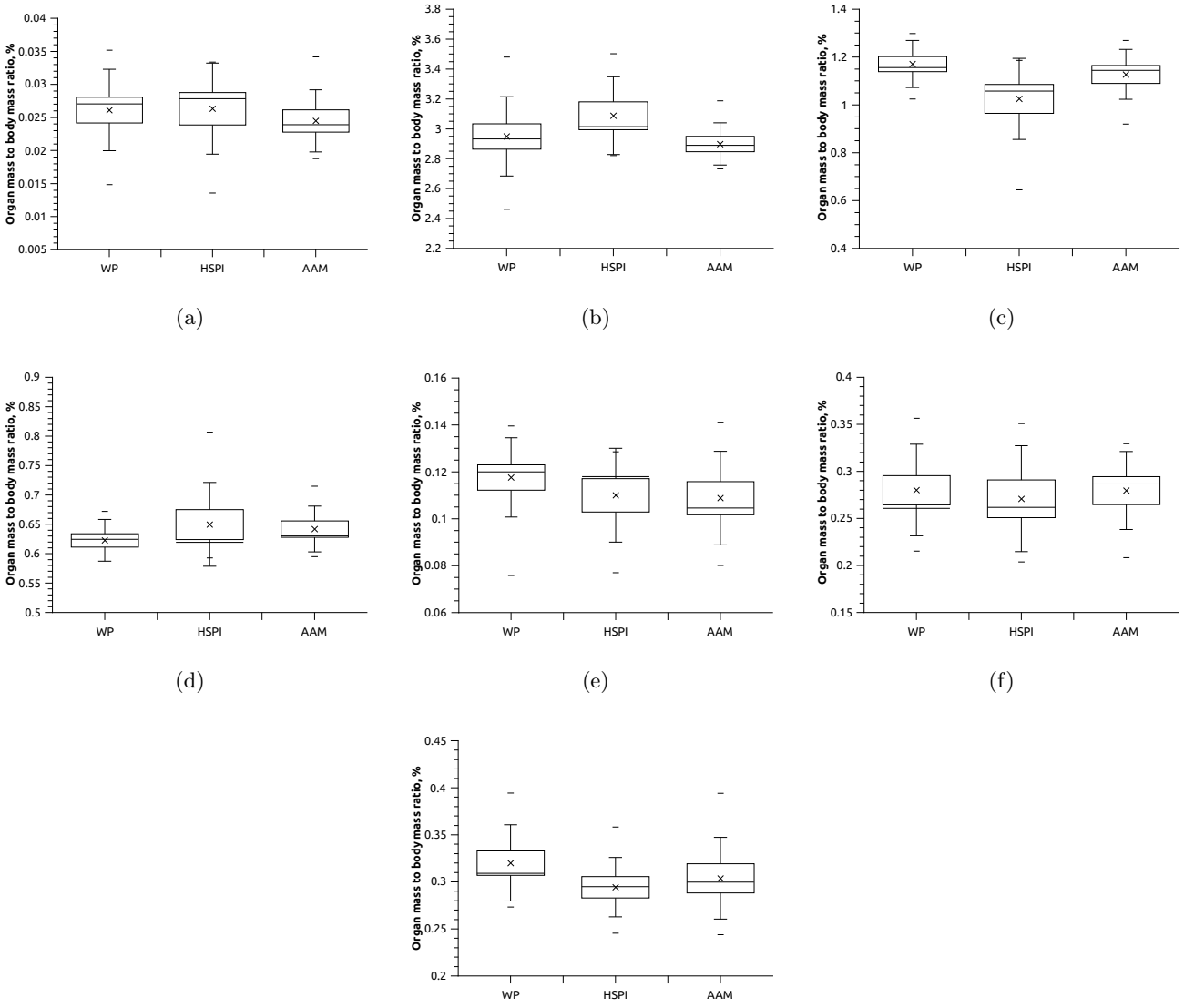

Figure A3. Relative weight of organs of animals receiving enterally whey protein (WP; N=10), hydrolysate of soy protein isolate (SPIH; N=8), a mixture of amino acids (AAM; N=8): (a) — adrenal glands, (b) — liver, (c) — testes, (d) — kidneys, (e) — thymus, (f) — spleen, (g) — heart. The following characteristics of the sample are graphically presented: the minimum and maximum of the sample with separate horizontal lines, the average — with a diagonal cross, a box — the interval ±SEM (standard error of the mean), a horizontal long line — the median, the whiskers — one standard deviation (±SD).

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Table A3. Sample size

Figure	Experimental day (see the axis of the figure)	N=		
		(a)	(b)	(c)
Fig. 2	1	9	8	9
	2	9	8	8
	3	9	8	9
	4	9	8	8
	5	8	8	7
	6	8	8	9
	7	9	8	9
	8	9	8	9
	9	9	8	9
	10	9	8	8
	11	9	8	8
		(a)	(b)	(c)
Fig. A1	0	9	8	10
	1	9	9	9
	2	10	9	9
	3	10	9	9
	4	10	9	8
	5	9	9	7
	6	8	9	9
	7	10	9	9
	8	10	9	9
	9	10	9	9
	10	10	9	9
	11	10	9	9
		(a)	(b)	(c)
Fig. A2	1	9	8	9
	2	9	8	8
	3	9	8	9
	4	9	8	8
	5	8	8	7
	6	8	8	9
	7	9	8	9
	8	9	8	9
	9	9	8	9
	10	9	8	8
	11	9	8	8
		Relative body weight gain, WP group)	Relative body weight gain, SPIH group	Relative body weight gain, AAM group
Fig. A4	1	9	8	9
	2	9	8	8
	3	9	8	9
	4	9	8	8
	5	8	8	7
	6	8	8	9
	7	9	8	9
	8	9	8	9
	9	9	8	9
	10	9	8	8
	11	9	8	8

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Table A4. Average feed consumption per animal, kg. The number of days since the first swimming test is indicated.

Group	1 day before the first swimming test	6 days after	13 days after	20 days after	27 days after
WP (1st cage)	0.0214	0.0236	0.0206	0.0216	0.022
WP (2nd cage)	0.0212	0.0226	0.0298	0.023	0.0236
SPIH (1st cage)	0.0224	0.025	0.022	0.0236	0.023
SPIH (2nd cage)	0.0206	0.0244	0.025	0.02425	0.0295
AAM (1st cage)	0.0226	0.0228	0.0194	0.022	0.028
AAM (2nd cage)	0.0214	0.0284	0.02675	0.02275	0.023

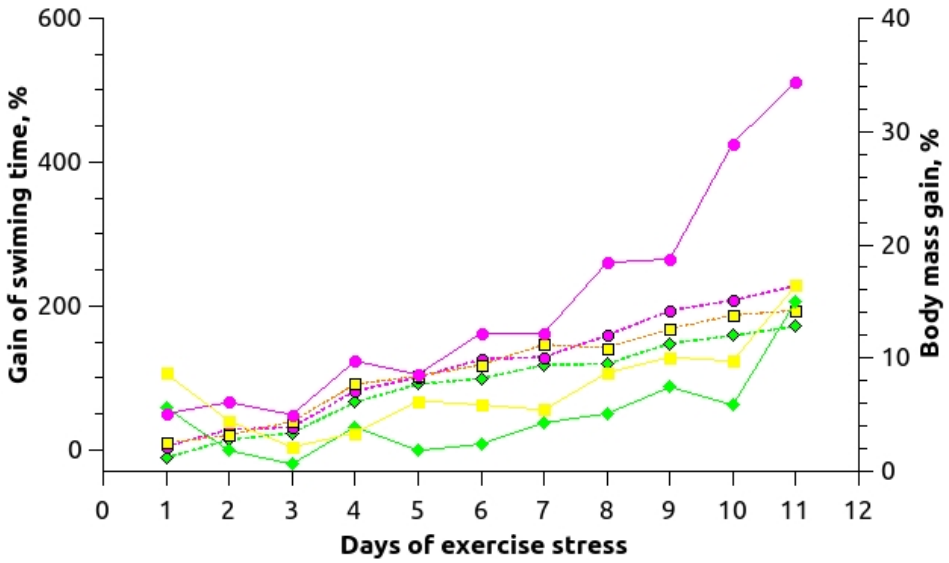

Figure A4. The relative increase in swimming time and body weight of rats that enterally received whey protein (yellow squares), soy protein isolate hydrolysate (pink circles), or a mixture of amino acids (green diamond). The average values of the increase in swimming time to exhaustion (solid lines) and body weight gain (dotted lines) relative to the "zero day" are indicated. The sample size for the relative increase in body weight, see Application Tab. A3, for the relative increase in swimming time, see Fig. 2.

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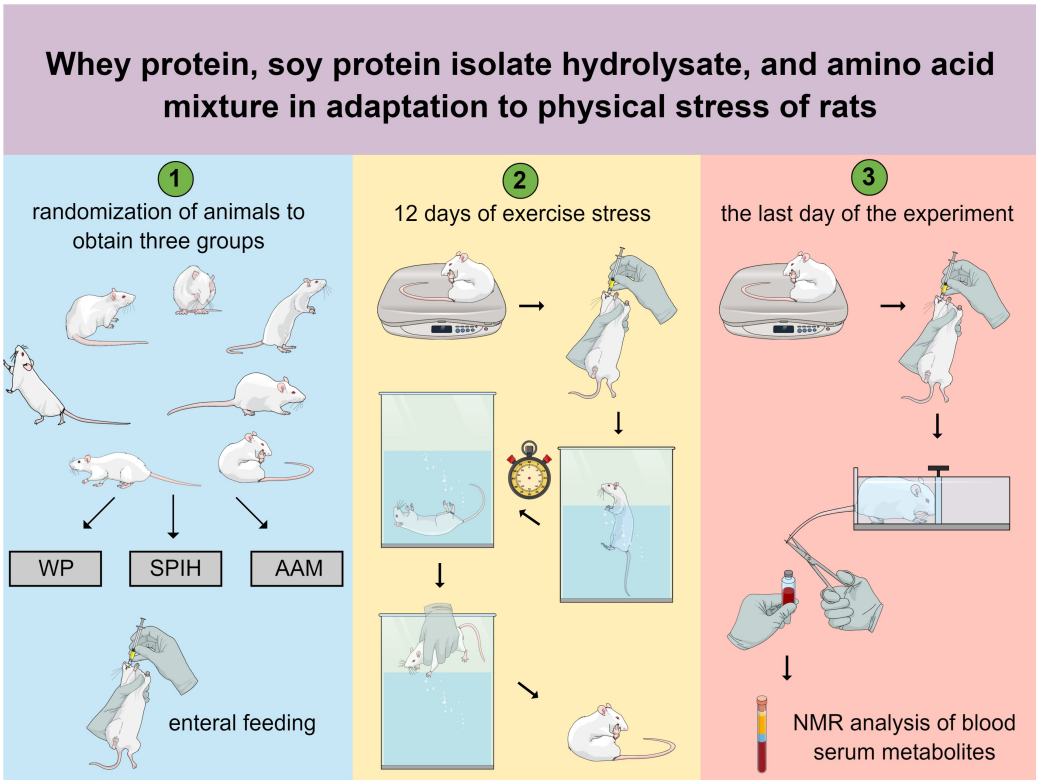

Figure A5. Graphic abstract

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Table A5. Identified blood serum metabolites

Metabolite
Formic acid
Cytidine
Tryptophan
Phenylalanine
Histidine
Tyrosine
Fumaric acid
2-Deoxycytidine
Uridine
Uracil
Allantoin
D-Glucose
D-Mannose
a-Glycerophosphocholine
Malate
L-Pyroglutamate
Proline
Serine
Betaine
Mannitol
Threonine
Glycerol
Glycine
Taurine
Choline
Acetyl-L-carnitine
L-Ornithine
Creatine
Lysine
2-oxoglutarate
Asparagine
Aspartic acid
Citric acid
Methionine
2-oxoisocaproate
Glutamine
Succinic acid
Pyruvic acid
Glutamic acid
Acetone
Acetic acid
Arginine
Alanine
Lactate
3-OH-butytrate
2-oxoisovalerate
3-Me-2-oxovalerate
Isobutytrate
Valine
Isoleucine
Leucine
2-OH-butytrate
2-OH-isovalerate

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Table A6. Distribution of peptides molecular weights of previously studied hydrolysates

Reference	Used proteolytic enzymes and preparations	Molecular weights of peptides, kDa
[7]	chymotrypsin, trypsin	5% 5–10 30% 1–5 65% < 1
[8], food product "Peptamen"	?	29% > 5 49% 1–5 21% 0.2–1 1% < 0.2
[10]	"Thermoase", "Bioprase", "Sumizyme"	8% >1 20% 0.5–1 72% <0.5