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THE EFFECT OF EXOGENOUSLY ACTING LYTIC ENZYMES AND FOOD-PROTEIN HYDROLYSATES ON IMMUNE SYSTEM

Summary of doctoral dissertation

Biomedical sciences, biology (01B),
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Introduction

Microbial lysis products (muropeptides and other peptidoglycan fragments, lipopolysaccharides, fungal cell wall glucans, etc.) are well known for their immunostimulatory actions (Werner, 1996; Alexander and Rietschel, 2001; Brown and Gordon, 2003). At least a half of the immunostimulants being applied in clinical practice are based on microbial cell-derived substances. Some characteristics of their action on functional immunocompetent cells, phagocytic ones including, are described (Nau and Eiffert, 2002; Alexander and Rietschel, 2001).

Technology of the immunostimulatory substances production is based on their extraction from the biomass by mechanic, ultrasonic or enzymatic methods. For the last-mentioned way extracellular or intracellular lytic enzymes (the latter are also called autolysins) are used.

Man hosts about 1 kg microorganisms located mainly in the gut lumen, which according to Bocci represents 'the neglected organ having a crucial immunostimulatory role' (Bocci, 1992). The biomass continuously supplies the host's organism with immunostimulatory microbial cell components. An intriguing alternative would be to find the ways to induce the release of microbial lysis products directly in the host's organism. One possible way might be the oral application of extracellular lytic enzymes. Another method by which the *in vivo* release of immunopotentiating compounds might be accomplished is the oral applications substances capable of stimulating the autolytic enzyme system of intestinal microorganisms.

A number of exogenously acting lytic enzymes differing in their specificity of action toward the main structural components of the microbial cell wall are known. Egg white lysozyme, proteolytic enzyme complexes (Wobenzym, Phlogenzym and etc.) are already used for enhancement of human and bacterial lysozyme, lysosubtilin and its derivative – of animal immune system (Sava, 1996; Kleine, 1997). As far as we know, no attempts have been made previously to employ the exogenously acting lytic enzymes as immunostimulants for reducing milk somatic cell count (SCC) in cows. So this study was aimed at evaluation of an enzymatic approach to reducing milk SCC. SCC is a commonly used measure of milk quality. From the immunological point of view, SCC is indirect indicator of the immune status of the cow and of the mammary gland in particular (Sordillo et al., 1997).

The microbial autolytic system is composed of a set of enzymes that cause damage to the essential components responsible for the integrity of cell wall. In bacteria these are peptidoglycan (murein hydrolases), with five classes of enzymes differing in their specificity for the various covalent linkages within the peptidoglycan. In fungi lytic endopeptidases, endoglucanases and chitinases are considered to be main autolytic enzymes (Smith et al., 2000; Кислухина, 2002; Bowman and Free, 2006; Salazar and Asenjo, 2007; Vollmer et al., 2008). Autolysins are essential for the growth and division of the microbial cell wall. The integrity of a microbe depends entirely on the precise control of autolytic system.

The autolytic system can be stimulated by pH, temperature and pressure, as well as chemicals such as mineral salts, EDTA, organic solvents or surfactants, with dissipation of the proton motive force being the most reliable mechanisms of microbial autolysis induction (Smith et al., 2000). However, it is difficult to imagine how these measures applied *in vitro* can be safely implemented *in vivo*.

A solution of the problem was facilitated by sighting that casein (milk protein) tryptic hydrolysate is capable of stimulating the microbial autolytic system (both *in vitro* and *in vivo*) and enhancing non-specific immunity (Biziulevičius et al., 2002; Biziulevičius et al., 2003). Casein tryptic hydrolysate – a mixture of bioactive peptides - carry out a wide range of functions (antimicrobial, immunostimulatory, antihypertensive, mineral binding, opioid, etc.).

Proteins that enter the organism as food components (food-proteins), after being hydrolyzed in the gastrointestinal tract by the digestive proteolytic enzymes, become a source not only of energy but also of a number of bioactive peptides beneficial to health (Tirelli et al., 1997; Schanbacher et al., 1997; Xu, 1998; Korhonen and Pihlanto, 2003; Meisel et al., 2003; Teschemacher, 2003; Aimutis, 2004; Hayes et al., 2007). Milk proteins are traditionally considered to be the most important source of bioactive peptides, although other animal as well as plant proteins are known to contain such peptides within their sequences. A mechanism of food-borne peptide antimicrobial action, as that of the immunostimulatory peptides, is unclear, although their basicity, hydrophobicity and structural characteristics are thought to play a crucial role (Hancock and Sahl, 2006; Sang and Blecha, 2008). In respect of this features the casein-borne antimicrobial peptides behave in somewhat similarity with the cationic antimicrobial peptides naturally occurring in the organism as components of the immune system. Although they have been studied extensively, the molecular mechanisms of action of antimicrobial peptides are not fully understood. It has been shown recently that transmembrane pore formation is not the only mechanism of microbial killing, and that antimicrobial peptides might affect the microbial cell in many other ways; for example, by inhibition of its wall and/or nuclei-acid synthesis, by activation of its autolytic enzyme system (Cudic and Otvos, 2002; McPhee and Hancock, 2005; Jenssen et al., 2006; Hancock and Sahl, 2006; Zaiou, 2007; Ginsburg and Koren, 2008).

Taking in mind the hypothesis (Biziulevičius, 2004), that some food-borne peptides may act as antimicrobials by activating the autolytic processes of the intestinal microflora *in situ* and thus cause a release of microbial lysis products, which are well-known immuno-enhancers, in this study some food-protein hydrolysates, were analysed for their capability to stimulate the autolytic system of microorganisms and immune system of animal.

The aim of this work was to establish the effect of exogenously acting lytic enzymes and food-protein hydrolysates on immune system.

The tasks of this work were as follows:

1. To evaluate the capability of food-protein casein, α-lactalbumin, β-lactoglobulin, ovalbumin and serum albumin hydrolysates to activate the autolytic system of naturally autolyzing and of naturally nonautolyzing microbial strains;
2. To evaluate the capability of studied food-protein to stimulate the phagocytosing capacity of peritoneum macrophages and blood monocytes and granulocytes;
3. To determine is there any correlation between antimicrobial and immunostimulatory activities of food-protein hydrolysates;

4. To evaluate the effect of the most active food-protein hydrolysate on inflammation;
5. To examine the effects of exogenously acting lytic enzyme preparations, lysozyme, lysosubtilin, their combinations and their combinations with vitamins, on milk somatic cell count in cows.

Practical value of the work

As a consequence of the emergence of antimicrobial resistance in medicine, an increased interest in identifying novel strategies to overcome infections is observed. Development of new antimicrobial agents is one of the possible solutions to get further spread of resistance under control. Agents that can benefcially affect the immune system, in adition to having direct antimicrobial activity, would be advantegous.

The results of this study shows, that exogenously acting lytic enzymes preparations, lysozyme and lysosubtilin, and food-protein hydrolysates, are effective antimicrobial and immunostimulating beneficial means, exactly such as required. Food-protein hydrolysates with last-mentioned activities are among the possible nutraceuticals. Food-protein hydrolysates may be incorporated in functional foods and pharmaceutical preparations. In this regard it is quite realistic to expect that products containing bioactive peptides isolated from enzymatic digest of food-protein will appear on the market in near future.

We also expect that the successful application of the exogenously acting lytic enzyme preparations to reducing milk SCC will find its place in a stockbreeding or milk industry practice.

Dissertation contents. The dissertation is written in Lithuanian and contains the following parts: Introduction, Materials and methods, Results, Discussion, Conclusions, List of publications. The work contains 5 tables, 6 figures, 1 scheme.

Materials and methods

Preparation of food-protein hydrolysates. Hydrolysates were prepared as described previously (Biziulevičius et al., 2002; Maehashi et al., 1999). Casein (Agrolitas Imex, Vilnius, Lithuania), α-lactalbumin, β-lactoglobulin, ovalbumin and serum albumin (all from Sigma, USA) were used. Each substrate protein was dissolved in distilled water of 80° C to 50 g/l concentration. The solution was cooled to 40° C, pH adjusted to 8.0 (pH 2.0 for pepsin was an exception) and hydrolyzed with trypsin, α-chymotrypsin, pepsin or pancreatin (Sigma) for 2 h under the following conditions: substrate concentration - 20 mg l^{-1}; enzyme-substrate ratio - 1 : 2500). Each hydrolysate was boiled for 20 min to inactivate the proteinase, then cooled and freeze-dried.

Preparation of microorganism biomass. Microorganism strains were purchased from JSC „Biosintezė“, Lithuania. Bacteria were prepared as described previously (Biziulevičius et al., 2002). Ten gram-positive bacterial strains, belonging to *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Streptomyces* species, nine gram-negative bacterial strains, belonging to *Escherichia*, *Methylococcus*, *Proteus* species as well as five fungal

(yeast) strains belonging to *Candida*, *Rhodotorula* and *Saccharomyces* species, were used. *Bacillus* species were grown in nutrient meat medium (1 % meat extract, 1 % peptone, 0,5 % NaCl) while the other were grown in brain heart infusion broth (0,35 % brain-heart extract, 2,2 % peptone, 0,2 % yeast extract, 0,2 % glucose, 0,5% NaCl) (Difco, USA). Culture flasks were shaken at 37°C until a post-logarithmic growth phase was reached. The cells were centrifuged, washed twice with 0.004 M-phosphate buffer of pH .2 and lyophilized.

Determination of antimicrobial activity. The antimicrobial activity was determined as described (Biziulevičius et al., 2002). The antimicrobial activity of food-protein hydrolysates, i.e. their ability to activate the autolytic enzyme system of the microbial cell (the autolysis activation index), was determined as follows. Suspensions of microbial cells were prepared in distilled water to an optical density at 520 nm of approximately 1.0, and were mixed with equal quantities of 4 mg ml^{-1} of the food-protein hydrolysate solution in distilled water. Controls were distilled water only. Optical densities at 520 nm of this mixture were measured at zero time (D_0) and after their exposure for 60 min at 37°C (D_{60}). The amount of autolysis (I) in both the study and control samples (I_b and I_k, respectively) was calculated using the formula:

$$I = [(D_0 - D_{60}) : D_0] \times 100\ \%$$

and then an autolysis activation index (K_A) for naturally autolyzing strains (I_k>0) was calculated as:

$$K_A = I_b : I_k .$$

whereas for naturally-nonautolyzing strains ($I_k = 0$) it was considered as $K_A = I_b$.

Experimental animals. The animal experiments as described below were coordinated with and allowed by Lithuanian Food and Veterinary office (licence for the use of laboratory animals No. 4, 2003-05-26). BALB/c mice of either sex, weighing 22-24 g, were maintained in an environment of controlled temperature (22±2)°C and food and water were provided *ad libitum*.

Determination of immunostimulatory activity. The immunostimulatory activity of food-protein hydrolysates, that is, their ability to enhance the phagocytosing capacity of the phagocytic cells, was evaluated using mice. The food-protein hydrolysate was given to mice (n=6 in each study groups) intragastrically once daily in a dose of 1.0 mg g^{-1} body weight dissolved in 0.5 ml of distilled water for five successive days. Control mice (a single group, n = 6) received equivalent volumes of distilled water. One day after food-protein hydrolysate solution (or distilled water) was given for the last time, the mice were subjected to procedures aimed at the assessment of the impact of oral food-protein hydrolysate on the phagocytosing capacity of peritoneal macrophages and blood monocytes and granulocytes. Quantitative evaluation of macrophage phagocytosing capacity was performed using a fluorometric assay while monocyte and granulocyte phagocytosing capacity was performed using a flow-cytometric assay.

Evaluation of macrophage phagocytosing activity by fluorometric method. Quantitative evaluation of macrophage phagocytosing capacity was performed using a fluorometric assay (Miliukiene` et al., 2003), but using pre-opsonized fluoresceinlabelled

Escherichia coli cells (Sigma) instead of the analogous ones of *Saccharomyces cerevisiae*. Briefly, the procedures were as follows. The mice were killed by rapid dislocation of the neck. No anaesthetic was used. The skin was then lifted upwards from the abdominal wall with forceps and cut, making a 50-mm long incision. Blood was taken by heart puncture and was heparinised with an intention to be further used for evaluation of phagocytosing capacity of blood phagocytic cell. Then 5 ml of Hanks balanced salt solution, containing 2 ml of heparin, was injected into the mouse peritoneal cavity. Peritoneal exudate cells were exsanguinated by peritoneal lavage. The cell suspension was incubated for 3 h at 37°C in Hanks balanced salt solution under 5 % CO_2 and 95 % air. At the end of incubation, mouse macrophages were firmly adhered to the glass surface. Next, the medium was removed and the adherent cells were washed vigorously three times with Hanks balanced salt solution to remove nonadherent cells. Cell viability was more than 98 %, as determined by trypan blue (Merck, Germany) exclusion. About 96 % of these adherent cells exhibited the morphological and staining characteristics (Giemsa stain was used) of macrophages. Fluorescein-labelled *E. coli* cells suspended in Hanks balanced salt solution were added to adherent phagocytic cells at a concentration of 5 x 10^5 cells ml^{-1}. The final bacteria to macrophage ratio was 400 : 1. After incubation at 37°C for 60 min, phagocytic cells were washed three times with phosphate-buffered saline (PBS) by aspiration to remove non-cell-ingested particles. A total of 100 ml of 0.04 % trypan blue (Merck) in PBS, pH 4.5 was added to the suspension and kept for 2 min to quench the unphagocytozed *E. coli* cells. Cells were then solubilized by the addition of 500 ml of 0.5 % Triton X-100 (BDH Chemicals, UK) in 0.01 M phosphate-buffered 0.15 M saline, pH 7.4. The solution was then transferred to a cuvette for measurement of the fluorescence intensity by a fluorescence spectrophotometer (MPF-4, Hitachi, Japan) with an excitation wavelength of 493 nm and an emission wavelength of 520 nm. The number of phagocytosed *E. coli* cells was evaluated in accordance with the relationship between the fluorescence intensity and the number of bacteria determined by applying the standard curve. The phagocytosing capacity was expressed as the number of ingested bacteria per cell.
A phagocytosing capacity enhancement index (K_I) was calculated as $KI = A_B/A_K$ where A_B and A_K are the phagocytosing capacity values of peritoneal macrophages of study and control mice, respectively.

Evaluation of phagocytosing activity by flow-cytometric method. Phagocytosing capacity of phagocytic cells (monocytes and granulocytes) of mouse blood was measured by applying a commercially available test kit (PHAGOTEST®, Germany). According to the instructions of the manufacturer the 100 µl of heparinzed blood was incubated with the 20 ml FITC-labelled *E.coli* bacteria at 37°C 10 min. The phagocytosis was stopped by placing the samples on ice and adding 100 µl quenching solution. After two washing steps with 3 ml washing solution erythrocytes were then removed by addition of 2 ml lysing solution. The cell suspension was washed with 3 ml washing solution after 20 min. incubation in room temperature. Then DNA staining solution was added. After 10 min. the phagocytosing capacity was measured by using flow cytometer FACS Calibur® (Becton Dickinson Biosciences, USA).
The phagocytosing capacity was expressed as the number of ingested bacteria per cell. A phagocytosing capacity enhancement index (K_I) was calculated as $KI = A_B/A_K$ where A_B and A_K are the phagocytosing capacity values of blood phagocytic cells of study and

control mice, respectively. Phagocytosing capacity was expressed as fluorescence intensity per phagocytic cell.

Evaluation of tryptic casein hydrolysate as a preventative measure against inflammation. Acute inflammation (paw edema) and contact hypersensitivity models were used to test a presumption that oral tryptic casein hydrolysate might be efficient for prevention of inflammatory processes.

Acute inflammation model. Acute inflammation was induced according (Jain et al., 2001). Tryptic casein hydrolysate was given to mice (n=6) intragastrically once daily in a dose of 1.0 mg g^{-1} body weight dissolved in 0.5 ml of distilled water for five successive days. Control mice (n=6) received equivalent volumes of distilled water. One day after tryptic casein hydrolysate solution (or distilled water) was given for the last time the acute inflammation was induced. The mice were challenged with an injection of 10 µl of 1 % carrageenan (Sigma, USA) solution into one hind paw. A day after the challenge the mice were killed, dissected and their spleen and thymus, the lymphoid organs responsible for the immune status of the animal in general, as well as hind paws (challenged and unchallenged ones) were weighted. At the same time, blood samples were collected by heart puncture for evaluation of blood variables (hematology), TNF-α and IL-10 concentrations.

Evaluation of blood variables. Hematologic analysis of mouse blood (white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, basophils, red blood cells, haemoglobin packed cell volume mean corpuscular volume mean cell haemoglobin, mean corpuscular haemoglobin concentration, red blood cell distribution width, platelets, mean platelet volume, angiotensin converting enzyme) was performed by using HEMAVET (CDC Technologies Inc., USA).

Determination of cytokines concentrations. Concentrations of cytokines TNF-α and IL-10 were determined in mice sera using ELISA kits for IL-10 and TNF-α and IL-10 assays BIOTRACK (Amersham Biosciences, England) according to the manufacturer's instructions.

Contact hypersensitivity reaction. Tryptic casein hydrolysate was given to mice (n=6) intragastrically once daily in a dose of 1.0 mg g^{-1} body weight dissolved in 0.5 ml of distilled water for five successive days. Control mice (n=6) received equivalent volumes of distilled water. A day before a five-day period of giving tryptic casein hydrolysate (or distilled water) mice were sensitised by application of 30 µl of 0.5 % 2.4-dinitrofluorobenzene (DNFB, Sigma, USA). One day after tryptic casein hydrolysate solution (or distilled water) was given for the last time the mice were challenged with an injection of 10 µl of 0.3 % DNFB into one hind paw. The same analyses as in case of acute inflammation model were performed.

Evaluation of exogenously acting lytic enzyme preparations, lysozyme and lysosubtilin, effect on reducing milk somatic cell count (SCC). Research was conducted at the research farm of the Lithuanian Veterinary Academy. Second to third lactation Lithuanian black and white cows with no clinical signs of mastitis were used.

The main criteria for their selection were a similar milk SCC [(700 ± 250) x 10^3 cells ml^{-1}) and a similar weight (550 ± 50 kg). Cows were maintained under usual industrial breeding conditions. Duration of the experiments were ten days. The experiments were performed according 1 scheme.

1 scheme. The scheme of evaluation of lysozyme and lysosubtilin effect on reducing milk somatic cell count.

Experiment	Group	Number of animal in each group (n)	Name of preparate and its dose
I	1 (control)	5	0
	2	5	5,0 mg kg wheigt^{-1} lysosubtilin
	3	5	10,0 mg kg wheigt^{-1} lysosubtilin
	4	5	20,0 mg kg wheigt^{-1} lysosubtilin
II	1 (control)	5	0
	2	5	50,0 mg kg wheigt^{-1} lysozyme
	3	5	100,0 mg kg wheigt^{-1} lysozyme
	4	5	200,0 mg kg wheigt^{-1} lysozyme
III	1 (control)	10	0
	2	10	200 mg kg wheigt^{-1} lysozyme
	3	10	20 mg kg wheigt^{-1} lysosubtilin
	4	10	200 mg kg wheigt^{-1} lysozyme + vitamins A, C, E
	5	10	20 mg kg wheigt^{-1} lysosubtilin + vitamins A, C, E
	6	10	200 mg kg wheigt^{-1} lysozyme + 20 mg kg wheigt^{-1} lysosubtilin
	7	10	200 mg kg wheigt^{-1} lysozyme + 20 mg kg wheigt^{-1} lysosubtilin + vitamins A, C, E

For all I and II experiments days once daily and day before the SCC in the evening milk of each cow were estimated by using a Somascope. Protein, fat and lactose quantities in milk were analysed for each cow by commonly applied methods a day before and a day after the ten-day period of giving lytic enzyme preparations.

In III experiment the SCC in the evening milk of each cow was estimated a day before giving enzymes, on fourth, seventh and tenth days of giving lytic enzyme preparations and on day 15.

Statistical analysis. Statistical analysis was performed by standard MS Excel program. Data are expressed as a mean of at least three independent experiments ± SD. Mean values were compared using Student's *t*-test. $P < 0,05$ was considered statistically significant. Correlation coefficient r_{xy} was determined for detection of relationship between two variables.

Results

Antimicrobial activity of food-protein hydrolysates. Food-proteins casein, α-lactalbumin, β-lactoglobulin, ovalbumin and serum albumin hydrolysed with enzymes trypsin, α-chymotrypsin, pancreatin and pepsin, acted antimicrobially *in vitro* towards all 24 microbial strains tested: autolysis of 20 naturally autolyzing strains was activated, with the autolysis activation index (K_A) ranging from 1.04 to 22.0 (Table 1), while autolysis was induced to values of 2.81–56.7 % in four naturally nonautolyzing strains (Table 2).

All tested food-protein hydrolysates were the most active against *Methylococcus* sp. M6-58, *E. coli* MRE-600 and *E. coli* O2 strains. Whereas autolytic systems of *B. subtilis* SK-52, *B. mesentericus* 66 ir *L. fermentii* 90TC4 were stimulated weakly. Casein hydrolysates were the most active stimulants, with a-lactalbumin, b-lactoglobulin, serum albumin and ovalbumin hydrolysates following (in decreasing order of activity). Similarly, if the influence of the proteolytic enzyme used is evaluated, the sequence is as follows: tryptic hydrolysates > peptic hydrolysates > chymotryptic hydrolysates > pancreatic hydrolysates.

Table 1. The intensity of naturally occuring autolysis (I_k) of microorganisms and autolysis activation indices (K_A) achieved when using various food-protein hydrolysates.

Microorganism	I_k (%)	K_A achieved when using: casein hydrolyzed with*				a-lactalbumin hydrolyzed with*				b-lactoglobulin hydrolyzed with*				ovalbumin hydrolyzed with*				serum albumin hydrolyzed with*			
		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Gram$^+$ bacteria																					
B.mesentericus 66	17,7	1,48	1,38	1,30	1,42	1,33	1,26	1,18	1,28	1,35	1,19	1,12	1,23	1,21	1,12	1,07	1,17	1,23	1,15	1,08	1,18
B.subtilis SK-52	16,0	1,45	1,35	1,28	1,39	1,31	1,24	1,16	1,27	1,32	1,17	1,10	1,20	1,19	1,10	1,04	1,15	1,20	1,13	1,06	1,16
B.subtilis 65-10	16,8	1,73	1,61	1,52	1,68	1,56	1,47	1,38	1,51	1,57	1,40	1,31	1,43	1,42	1,31	1,25	1,37	1,44	1,35	1,26	1,38
B.subtilis 65-42	18,4	2,78	2,59	2,45	2,67	2,50	2,36	2,22	2,42	2,53	2,25	2,11	2,31	2,28	2,11	2,00	2,20	2,31	2,17	2,03	2,22
B.subtilis 65-1482	17,6	1,94	1,80	1,71	1,86	1,75	1,65	1,55	1,69	1,77	1,57	1,47	1,61	1,59	1,47	1,40	1,53	1,61	1,51	1,42	1,55
Bifidobacterium sp.	8,0	8,69	8,08	7,64	8,34	7,82	7,39	6,96	7,56	7,89	7,04	6,60	7,21	7,13	6,60	6,26	6,87	7,21	6,78	6,34	6,95
L.fermentii 90TC4	26,3	1,57	1,46	1,38	1,51	1,41	1,33	1,26	1,37	1,43	1,27	1,19	1,30	1,29	1,19	1,13	1,24	1,30	1,22	1,15	1,26
L.plantarum 8PA3	7,0	2,99	2,78	2,63	2,87	2,69	2,54	2,39	2,60	2,72	2,42	2,27	2,48	2,45	2,27	2,15	2,36	2,48	2,33	2,18	2,39
Gram$^-$ bacteria																					
E.coli K12	14,5	2,41	2,24	2,12	2,31	2,17	2,05	1,93	2,09	2,19	1,95	1,83	2,00	1,98	1,83	1,74	1,90	2,00	1,88	1,76	1,93
E.coli M17	5,1	6,31	5,86	5,55	6,06	5,68	5,36	5,05	5,49	5,74	5,11	4,80	5,24	5,17	4,80	4,54	4,98	5,24	4,92	4,61	5,05
E.coli MRE-600	2,5	15,04	13,99	13,23	14,44	13,54	12,78	12,03	13,08	13,69	12,18	11,43	12,48	12,33	11,43	10,83	11,88	12,48	11,73	10,98	12,03
E.coli O2	1,0	11,40	10,60	10,03	10,94	10,26	9,69	9,12	9,92	10,37	9,23	8,66	9,46	9,35	8,66	8,21	9,00	9,46	8,89	8,32	9,12
M. capsulatus 122	5,5	2,95	2,74	2,59	2,83	2,66	2,51	2,36	2,57	2,68	2,39	2,24	2,45	2,42	2,24	2,12	2,33	2,45	2,30	2,15	2,36
M.capsulatus 170	6,2	4,40	4,09	3,87	4,22	3,96	3,74	3,52	3,83	4,00	3,56	3,34	3,65	3,61	3,34	3,17	3,48	3,65	3,43	3,21	3,52
Methylococcus sp. M6-58	0,5	22,00	20,46	19,36	21,12	19,80	18,7	17,60	19,14	20,02	17,82	16,72	18,26	18,04	16,72	15,84	17,38	18,26	17,16	16,06	17,60
P. vulgaris	5,2	2,02	1,87	1,78	1,94	1,82	1,72	1,62	1,76	2,00	1,64	1,54	1,67	1,66	1,54	1,45	1,60	1,68	1,58	1,47	1,62
Fungi (yeasts)																					
C.salmonicola 779	8,6	2,57	2,39	2,26	2,47	2,31	2,18	2,06	2,24	2,34	2,08	1,95	2,13	2,11	1,95	1,85	2,03	2,13	2,00	1,88	2,06
C.tropicalis 909	4,9	2,88	2,67	2,53	2,76	2,59	2,45	2,30	2,51	2,62	2,33	2,18	2,39	2,36	2,19	2,07	2,28	2,39	2,25	2,10	2,30
R.aurantiaca 528	8,4	2,98	2,77	2,62	2,86	2,68	2,53	2,38	2,59	2,71	2,41	2,26	2,47	2,44	2,26	2,15	2,35	2,47	2,32	2,18	2,38
S.cerevisiae 12	1,9	4,79	4,45	4,21	4,59	4,31	4,07	3,82	4,17	4,36	3,88	3,64	3,98	3,93	3,64	3,45	3,78	3,98	3,74	3,50	3,83

Results are presented as the averages of six replicates. * The enzymes: 1, trypsin; 2, a-chymotrypsin; 3, pancreatin; 4, pepsin.

Table 2. The influence of various food-protein hydrolysates on the intensity of autolysis (I_b) of naturally nonautolyzing ($I_k = 0$) microorganisms

Microorganism	I_b (%) achieved when using:																			
	casein hydrolyzed with*				a-lactalbumin hydrolyzed with*				b-lactoglobulin hydrolyzed with*				ovalbumin hydrolyzed with*				serum albumin hydrolyzed with*			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
B.mesentericus 1	19,9	18,51	17,51	19,10	17,91	16,92	15,92	17,31	18,11	16,12	15,12	16,52	16,32	15,12	14,33	12,42	16,52	15,52	14,53	15,92
	8,2	7,63	7,22	7,87	7,38	6,97	6,56	7,13	7,46	6,64	6,23	6,81	6,72	6,23	5,90	6,48	6,81	6,40	5,60	6,56
C. lambica 522																				
	3,9	3,63	3,42	3,74	3,51	3,32	3,12	3,39	3,55	3,16	2,96	3,24	3,20	2,96	2,81	3,08	3,24	3,04	2,85	3,12
M. acidoxilos																				
	56,7	52,73	49,90	54,43	51,03	48,20	45,36	49,33	51,60	45,93	43,09	47,06	46,50	43,10	40,82	44,80	47,06	44,23	41,40	45,36
S. albogriseolus																				

Results are presented as the averages of six replicates. * The enzymes: 1, trypsin; 2, a-chymotrypsin; 3, pancreatin; 4, pepsin.

Immunostimulatory activity of food-protein hydrolysates. When given to mice *per os*, all of the food-protein hydrolysates enhanced the phagocytosing capacity of peritoneal macrophages, with a K_I of 1.10–1.39 (Table 3).

Table 3. The influence of various food-protein hydrolysates when given to mice *per os* on the phagocytosing capacity of their peritoneal macrophages and blood monocytes and granulocytes.

Hydrolysates	Macrophage phagocytosing capacity (K_I)	Blood monocytes and granulocytes phagocytosing capacity (K_I)
Casein hydrolyzed with		
trypsin	1,39	1,34
pepsin	1,34	1,30
a-chymotrypsin	1,32	1,29
pancreatin	1,27	1,20
a-Lactalbumin hydrolyzed with		
trypsin	1,30	1,27
pepsin	1,26	1,25
a-chymotrypsin	1,25	1,21
pancreatin	1,20	1,14
b-Lactoglobulin hydrolyzed with		
trypsin	1,29	1,27
pepsin	1,26	1,20
a-chymotrypsin	1,23	1,16
pancreatin	1,19	1,13
Serum albumin hydrolyzed with		
trypsin	1,25	1,23
pepsin	1,23	1,19
a-chymotrypsin	1,20	1,15
pancreatin	1,18	1,13
Ovalbumin hydrolyzed with		
trypsin	1,24	1,18
pepsin	1,21	1,16
a-chymotrypsin	1,20	1,16
pancreatin	1,16	1,10

Interestingly, the greater the ability of the hydrolysate to stimulate the microbial autolytic system (K_A), the higher the value of K_I. Moreover, a close correlation was observed between the K_A of food-protein hydrolysates in regard to each of the 24 microorganisms tested and K_I (Fig. 1). Based on the latter result in respect to the immunostimulatory effects of microbial lysis products, we presume that the immunostimulatory activity of foodprotein hydrolysates is a consequence of their antimicrobial activity; that is, $K_I = f(K_A)$.

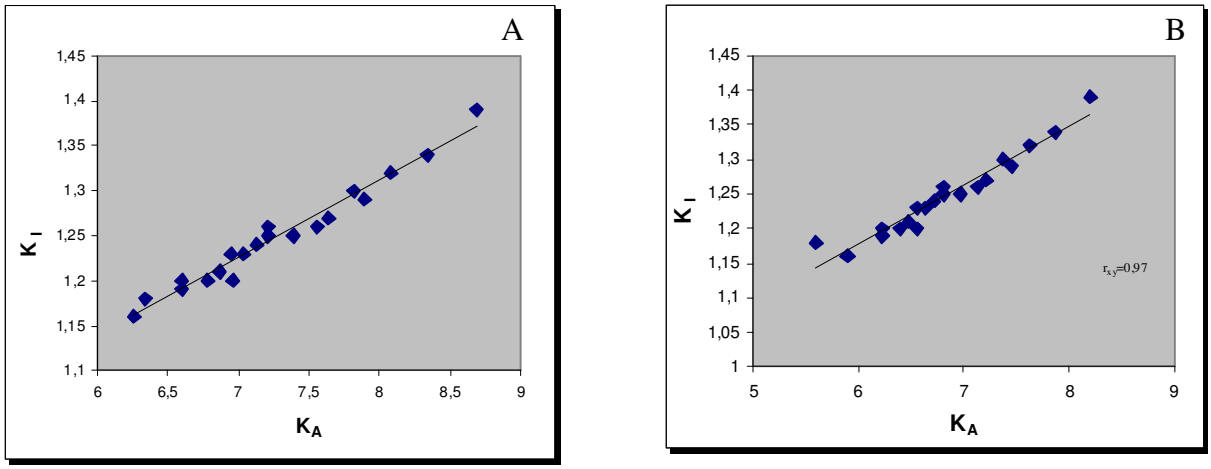

Fig. 1. Relationship between the antimicrobial activity (K_A) and the immunostimulatory activity (K_I) of food-protein hydrolysates (A: in regard to *Bifidobacterium sp.*; B: in regard to *C. lambica*) compiled from the results presented in Tables 1–3.

Effect of tryptic casein hydrolysate on inflammation. Acute inflammation (paw edema) and contact hypersensitivity models were used to test a presumption that oral tryptic casein hydrolysate (the latter was the most active stimulant of microorganisms autloytic system and phagocytosing capacity of phagocytic cells) might be efficient for prevention of inflammatory processes. The results are given in Figs. 2 - 3.

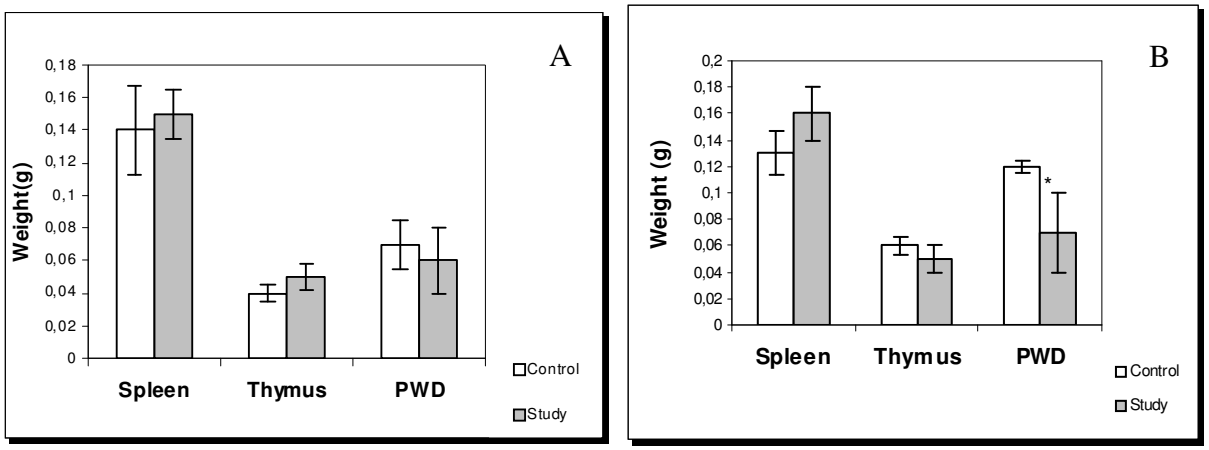

Fig. 2. Spleen and thymus weights, and weight difference of challenged and unchallenged hind paws (paw weight difference; PWD) of study and control mice observed in acute inflammation (paw edema) model (A) and in contact hypersensitivity model (B). n=6 in each group. *Data are significantly different ($p<0.05$) from the control group.

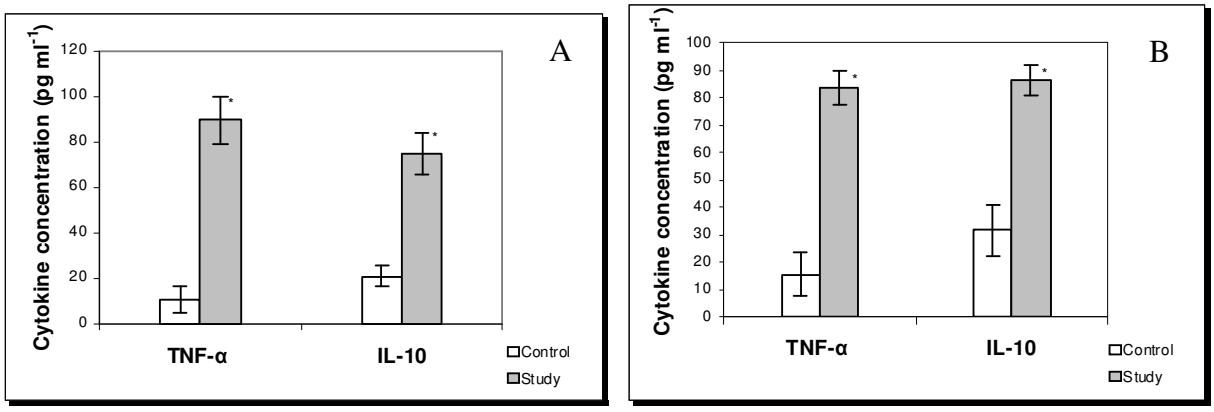

Fig. 3. TNF-α and IL-10 concentrations in sera of study and control mice observed in acute inflammation (paw edema) model (A) and in contact hypersensitivity model (B). n=6 in each group. *Data are significantly different ($p<0.001$) from the control group.

A significant decrease in weight difference ($p<0.05$) of challenged and unchallenged hind paws of study versus control mice, that was observed in a contact hypersensitivity model (Fig. 2B), but not in an acute inflammation model (Fig. 2A), was the only result in favour of the presumption. In a contact hypersensitivity model the paw edema was inhibited 41.7 %. No significant differences as in regard to spleen and thymus weights of study and control mice in both the models were observed (Fig. 2), nor such differences were found when hematological indices were compared (Table 4 and 5).

Table 4. Hematological indices in blood of study and control mice observed in acute inflammation model. There is no significant differences.

Hematological index	Group (*n*=6)	
	Control	Study
WBC ($x10^9 l^{-1}$)	3.54±1.02	4.77±1.17
NE ($x10^9 l^{-1}$)	0.43±0.13	0.69±0.27
LY ($x10^9 l^{-1}$)	2.39±0.83	3.33±0.89
MO ($x10^9 l^{-1}$)	0.42±0.08	0.54±0.13
EO ($x10^9 l^{-1}$)	0.03±0.005	0.03±0.01
BA ($x10^9 l^{-1}$)	0.002±0.0004	0.001±0.001
RBC ($x10^{12} l^{-1}$)	8.45±1.01	10.06±1.49
Hgb ($g l^{-1}$)	112.3±5.7	117.5±5.2
PCV ($x10^{-2} l l^{-1}$)	39.03±3.60	45.5±7.45
MCV ($x10^{-15} l l^{-1}$)	46.37±4.84	46.82±0.50
MCH ($x10^{-12} g l^{-1}$)	12.73±1.48	12.63±0.52
MCHC ($x10 g l^{-1}$)	29.4±2.47	27.53±1.36
RDW (%)	17.75±3.81	21.42±1.47
PLT ($x10^{11} l^{-1}$)	1.18±0.097	1.06±0.049
MPV ($x10^{-15} l l^{-1}$)	43.2±9.3	38.5±5.2

Table 5. Hematological indices in blood of study and control mice observed in contact hypersensitivity model. There is no significant differences.

Hematological index	Group (*n*=6)	
	Control	Study
WBC ($x10^9 l^{-1}$)	3.54±1.02	4.77±1.17
NE ($x10^9 l^{-1}$)	043±0.13	0.69±0.27
LY ($x10^9 l^{-1}$)	2.39±0.83	3.33±0.89
MO ($x10^9 l^{-1}$)	0.42±0.08	0.54±0.13
EO ($x10^9 l^{-1}$)	0.03±0.005	0.03±0.01
BA ($x10^9 l^{-1}$)	0.002±0.0004	0.001±0.001
RBC ($x10^{12} l^{-1}$)	8.45±1.01	10.06±1.49
Hgb ($g l^{-1}$)	112.3±5.7	117.5±5.2
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RDW (%)	17.75±3.81	21.42±1.47
PLT ($x10^{11} l^{-1}$)	1.18±0.097	1.06±0.049
MPV ($x10^{-15} l l^{-1}$)	43.2±9.3	38.5±5.2

Moreover, significantly higher concentration of cytokines TNF-α and IL-10 ($p<0.001$) were observed in sera of study versus control mice in any of the inflammatory models used (Fig. 3). In contact hypersensitivity model concentration of cytokine TNF-α was 5.4 and IL-10 – 2.7 times higher when compared with control groups.

The effect of exogenously acting lytic enzyme preparations, lysozyme and lysosubtilin, on reducing milk somatic cell count (SCC). The highest lysosubtilin (20 mg kg wt^{-1}) and lysozyme (200 mg kg wt^{-1}) doses then given to cows with feed once daily for ten successive days resulted in a statistically significant ($p<0.05$) reduction of milk SCC (Fig. 4).

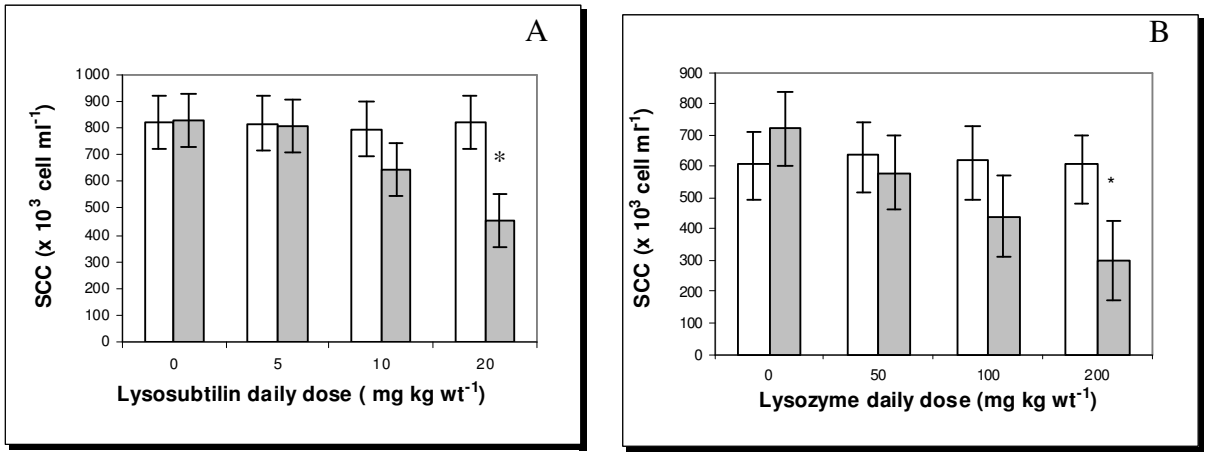

Fig. 4. Effect of lysosubtilin (A) and lysozyme (B) on SCC in cows when given at different doses. There were five cows in each group. Data are given as mean ± SD. □ - before giving lysosubtilin or lysozyme; ■ – after a ten day period of giving lysosubtilin or lysozyme.

No significant changes in protein, fat and lactose quantities in milk influenced by enzymes highest doses application were observed (Fig. 5).

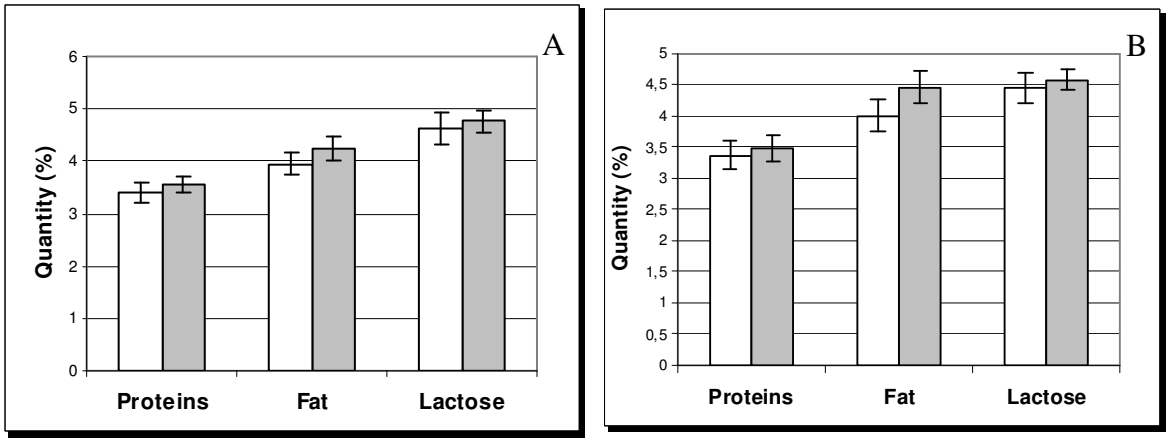

Fig. 5. Effect of lysosubtilin (A) and lysozyme (B) on quantity of proteins, fat and lactose when given to cows at doses 20 mg kg wt^{-1} and 200 mg kg wt^{-1}. There were five cows in each group. Data are given as mean ± SD. □ - before giving lysosubtilin or lysozyme; ■ – after a ten day period of giving lysosubtilin or lysozyme.

Results of comparative field trial are given in Fig. 6. After four-, seven-, and ten-day periods of giving enzymes (with or without supplemental vitamins) a significant reduction of SCC ($p<0.001$) was observed in milk of cows that received a combination

of lysozyme with vitamins, while on the tenth day a significant reduction of SCC ($p< 0.001$) was also observed in milk of cows that received lysozyme and lysosubtilin (each alone; without supplemental vitamins) or lysosubtilin in combination with vitamins. An increase in SCC on the fourth day in milk of cows that received preparations other than lysozyme with vitamins when compared to the untreated control stands in contrast to these results. At the end of the trial (on the 15th day) SCC in milk of cows of any of the study groups was significantly lower ($p<0.001$) when compared with that of the control group. As for SCC level in milk of the control group, in general, it was quite consistent during the treatment period, but it was almost double that on the 15th day.

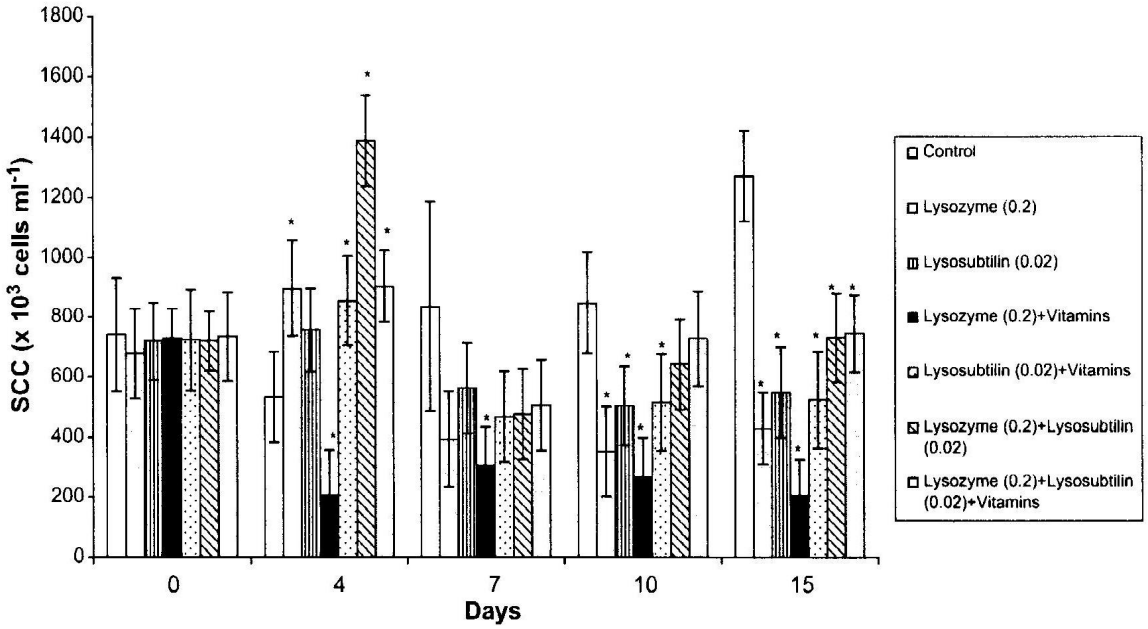

Figure 6. Effect of oral application of enzymes (and vitamins) on milk somatic cell count (SCC) in cows. Cows were given lysozyme and/or lysosubtilin (with or without supplemental vitamins) once daily with feed for ten successive days. The daily doses of the enzymes (g kg wt^{-1}) are shown on an insert (in brackets), while vitamins A, C and E were given at doses twice as high as required for nutritional adequacy. There were ten cows in each group. Data are given as mean ± SD. *Data are significantly different ($p< 0.001$) from the control group.

Discussion

As far back as 1981, Olga Kislukhina showed that tryptic casein hydrolysate may act as a stimulant of the microbial autolytic system (Kislukhina et al., 1981). This observation was based on examination of microbial lysis processes (Kislukhina et al., 1990) as well as on the results of earlier studies on agents stimulating microbial autolysis. Although Kislukhina's observation (Kislukhina et al., 1981) included a speculation that hydrolysates of other food proteins might also activate the autolysis of microorganisms, this possibility remained unexplored until now. Previous studies revealed that antimicrobial treatment of newborn calf colibacillosis with tryptic casein hydrolysate yields a significant improvement of a number of blood and serum indices (γ-globulin and sulphhydryl groups quantity, total protein quantity, bactericidal activity).

In the present study, twenty food-protein hydrolysates were analysed and all were found to stimulate the autolytic system of all twenty naturally autolyzing as well as four naturally nonautolyzing microbial (bacterial and fungal) strains tested. The autolysis

activation/induction level in various microorganisms was also very different. Thus, the differences in the susceptibility of microbial strains to the food-protein hydrolysates used indicate that the nature of food protein and proteolytic enzyme, as well as the nature of the microorganism itself, are all important. Nevertheless, the ability to stimulate the microbial autolytic system is likely to be the common denominator in food-protein hydrolysates. It must, however, be noted that this postulate is based on *in vitro* studies only, and whether the food-protein hydrolysates express such an ability in *in vivo* systems is still to be proved.

The immunostimulatory effects of food-protein-derived biologically active peptides so far studied are achieved mainly through the enhancement of phagocytic cell functioning. These cells are part of nonspecific immunity, and their activities, especially those of macrophages, are a key element of immune surveillance. In this study all food-protein hydrolysates tested, when given to mice *per os* were shown to enhance the phagocytosing capacity of peritoneal macrophages and blood monocytes and granulocytes (Table 3).

A close correlation was observed between the ability of food-protein hydrolysates to stimulate the microbial autolytic system and phagocytosing capacity of phagocytic cells. Based on these result in respect to the immunostimulatory effects of microbial lysis products, we presume that the immunostimulatory activity of food-protein hydrolysates is a consequence of their antimicrobial activity.

The hypothesis, that at least some food-borne peptide may activate the autolytic processes of the intestinal microflora *in situ* and thus result in the release of microbial lysis products, these being finally accountable for the immunostimulatory effects achieved by consumption of food proteins (Biziulevičius, 2004), was not only verified by results of this work but also allowed to rice 'cause an effect' theory of bifunctionality: food-protein enzymatic hydrolysates possess both antimicrobial and immunostimulatory activities. This theory could be used in health-related practices such as creating of functional food. Several food-protein hydrolysates and/or individual peptides derived from them have already been proved to be a benefit to health, and have been claimed as potential components of functional foods or pharmaceutical preparations. According to Chandra (1996), 'the era of nutritional manipulation of the immune system has finally dawned and it brings with it the promise of using diet and nutrition as innovative powerful tools to reduce illness and death caused by infection'.

The tryptic casein hydrolysate (the most active stimulant of microorganisms autolytic system and phagocytosing capacity of phagocytic cells) seemed to be ineffective mean for prevention of experimentally induced inflammation. The main cause of such inefficiency could be cross-talking between activated phagocytes and microbial lysis products (Labro, 2000; Ginsburg, 2002). Phagocytic cells and microbial lysis products are both considered a double-edged sword, each playing though a separate but fundamental role in the pathophysiology of inflammation. Interactions between the two are very complex and often lead to imbalance of proinflammatory and anti-inflammatory cytokines (Labro, 2000; Ginsburg, 2002). As for results of the present study regarding cytokines, we do not think that a systemic induction by oral tryptic casein hydrolysate of TNF-α and IL-10 observed both in an acute inflammation and contact hypersensitivity models (Fig. 3), belong to harmful effects, that could damage inflamed animal organism. Rest results on inflammation, namely the significant decrease in paw weight difference (a positive result), observed in contact hypersensitivity model,

but not in paw edema experiment and no significant differences in weights of spleen and thymus (Fig. 2) as well as in haematological indices of study and control mice in both the experiments, all come in support to innocuousness of oral tryptic casein hydrolysate at inflammation.

The second part of this work is concerned with the application of exogenously acting lytic enzymes to reducing somatic cell count (SCC). Milk somatic cells are mostly cells of the immune system (lymphocytes, macrophages, neutrophils, epithelial cells, etc.). They are part of the natural defence mechanism. SCC is a commonly used measure of milk quality. In the healthy lactating mammary gland, the number of somatic cells is usually less than 150 x 10^3 ml^{-1} of milk, however, in the inflamed one, it can increase to several millions ml^{-1} of milk (Harmon 1994, Booth 2000). Thus, SCC is an indicator of the inflammatory response to an intramammary infection or another trigger of the immune system (Rivas et al. 2001, Schukken et al. 2003, Green et al. 2004, Robert-Granie et al. 2004, de Haas et al. 2005, Hillerton & Berry 2005). Since the benefits in reducing milk SCC (less mastitis, increased yields, better milk quality, better milk products, reduced risk to human health) have been proved to be profitable (Booth 2000), new ways to improve the achieved result are being searched for. In recent years, considerable research effort has focused on enhancing the natural defence mechanisms of the mammary gland.

Recent advances in bovine immunology indicate the increasing interest in using immunomodulators to beneficially affect animal health as a possible tool for reducing milk SCC. In this regard immunostimulatory effects arising from the oral application of enzyme preparations when used for antimicrobial treatment (Biziulevičius et al., 1997, Biziulevičius et al., 2003) allows us to predict that these enzymes might be the prospective candidates. As far as we know, no attempts have been made previously to employ the enzymes as immunostimulants for reducing milk SCC in cows. So in this study the efficacies for reduction of milk SCC of both lysosubtilin and lysozyme, a combination thereof and a combination of the enzyme preparations (each alone and in combination) with vitamins as possible coimmunostimulants were evaluated.

Lysosubtilin – enzyme complex, acetone-precipitated preparation of lytic enzymes (two lytic endopeptidases) derived from B. subtilis SK-52, possessing a broad spectrum of antimicrobial action (Biziulevičius et al., 2002).

Lysozyme (lytic carbohydrase) is distributed throughout the animal, plant and microbial kingdoms. It is amongst the enzymes that are both the best studied and most widely used (Proctor et al., 1988; Fuglsang et al., 1995; Jolle`s, 1996; Sava, 1996; Malinowski, 2001).

Lysosubtilin and lysozyme act through disruption of microbial cell wall and releasing lysis products (muropeptides, lipopolysaccharides, etc.), that are responsible for immunostimulatory effects.

The results of the study (Fig. 4-5) show that application of highest doses of lysosubtilin (20 mg kg wt^{-1}) and lysozyme (200 mg kg wt^{-1}) resulted in a statistically significant ($p< 0.05$) reduction of milk SCC in cows but only lysozyme reduced SCC until permissible standard. Lysosubtilin reduced SCC an average 43.9 % and lysozyme – 50.4 %. No negative impact to quantities of milk proteins, lactose and fat was observed. It must be said that these effects were achieved at quite different conditions (the study with lysosubtilin was performed in early spring, whilst the study with lysozyme was

performed in autumn) and thus the results are hard to compare. For this reason comparative study was performed (Fig. 6).

An average 40.6% and 58.5% reduction of SCC was observed on the tenth day of the trial in milk of cows that were given lysosubtilin and lysozyme (individually), respectively. The results achieved are in a rather close correlation with the previous ones. It appears that lytic carbohydrases (represented in this study by lysozyme) have greater potential for future studies than lytic endopeptidases (represented in this study by lysosubtilin).

As for beneficial effects resulting from the application of a combination of lysosubtilin and lysozyme (with or without vitamins), these were not as expected. In contrast, supplementation of lysozyme (but not of lysosubtilin) with vitamins A, C and E gave very good results that were observed throughout the trial. Moreover, a combination of lysozyme with vitamins was the only positive effect observed on the fourth day of the trial. 64,3 % reduction of milk somatic cell count was achieved on the tenth day of this combination oral application. As for the reason for the increase in SCC on the fourth day in milk of cows that received other preparations, it remains unclear, though a possibility is that this was the result of microbial lysis products' (depending on various factors) acting either as immunostimulants or immunosuppresors (Ginsburg 2002). Taken on the other hand, it is even harder (if at all possible) to explain what caused the increase in SCC in milk of cows of the control group at the end of the trial (on the 15th day). In any case, the latter observation confirms overall positive effect of enzyme application, rather than comes in contradiction to it.

The results of this work revealed, that exogenously acting lytic enzymes or food-protein hydrolysates inducing autolytic system of microorganisms have antimicrobial activity and also act as immunostimulants. Though food-protein casein tryptic hydrolysate have no anti-inflammatory effect, but exogenously acting enzymes efficiently reduced somatic cell count, which is an indicator of the inflammatory response. So we propose that lytic enzymes lysosubtilin and lysozyme and food-protein hydrolysates are effective antimicrobials and immunostimulants.

Conclusions

1. Food-proteins casein, α-lactalbumin, β-lactoglobulin, ovalbumin and serum albumin hydrolysed with trypsin, chymotrypsin, pancreatin and pepsin activated the autolytic system of naturally autolyzing microbial strains. Autolysis activation index (K_A) ranged from 1.04 to 22.0.

2. Studied food-protein hydrolysates were capable to activate the autolytic system of naturally nonautolyzing microbial strains. The intensity of autolysis (I_b) ranged from 2.81 % to 56.7 %.

3. Studied food-protein hydrolysates enhanced the phagocytic activity of phagocytic cells. The phagocytosing capacity of peritoneal macrophages was stimulated from 1.16 to 1.39 times. The phagocytosing capacity of blood monocytes and granulocytes was enhanced from 1.10 to 1.34 times.

4. A close correlation ($r_{xy}>0.9$) was observed between the food-protein hydrolysates capability to stimulate the microbial autolytic system and phagocytosing activity of phagocytic cells.

5. The tryptic casein hydrolysate had no effect on prevention of inflammation, but in contact hypersensitivity model the paw edema was inhibited 41.7 %. In this case, concentration of cytokine TNF-α was 5.4 and IL-10 – 2.7 times higher when compared with control groups.

6. Exogenously acting lytic enzyme preparation lysozyme combined with vitamins A, C and E, most effective reduced milk somatic cell count in cows. 64.3 % reduction of milk somatic cell count was achieved on the tenth day of this combination oral application.

List of publications

1. Biziulevičius, G.A., Kazlauskaitė, J., Lukauskas, K., Ramanauskienė, J., Sederevičius, A. (2003) An enzymatic cow immunity-targeted approach to reducing milk somatic cell count: 1. A preliminary study using lysosubtilin. Food and Agricultural Immunology, 15, 289-292.
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Reziumė

Šio darbo rezultatai rodo, kad maisto baltymų, t.y. α-laktalbumino, β-laktoglobulino, ovalbumino ir serumo albumino, hidrolizatai, gauti panaudojus virškinimo fermentus tripsiną, chimotripsiną, pankreatiną ir pepsiną, stimuliavo mikroorganizmų, priklausančių skirtingoms taksonominėms grupėms, autolizinę sistemą. Skyrėsi tik poveikio stiprumas tiek mikroorganizmo kamieno, tiek baltymo bei virškinimo fermento atžvilgiais. Įvertinus maisto baltymų hidrolizatų gebėjimą stimuliuoti organizmo imuninę sistemą, nustatyta, kad fagocitinių ląstelių aktyvumą stimuliuoja visi maisto baltymų hidrolizatai. Aktyvinami buvo tiek pilvaplėvės makrofagai, tiek kraujo fagocitinės ląstelės (monocitai ir granuliocitai). Statistinė rezultatų analizė parodė, kad tarp maisto baltymų hidrolizatų antimikrobinio ir imunostimuliuojančio aktyvumų egzistuoja tiesioginis ryšys (r_{xy}> 0,9), t.y. kuo labiau maisto baltymų hidrolizatas pajėgus skatinti mikroorganizmų autolizę, tuo stipresnis jo poveikis imuninei sistemai.

Šio darbo rezultatai patvirtino hipotezės teisingumą ir leido sukurti mokslinę teoriją: *maisto baltymams, suardytiems virškinimo proteoliziniais fermentais iki peptidų, būdingas bifunkcinis (antimikrobinis/ imunostimuliuojantis) veikimas. Baltymų imunostimuliuojantis aktyvumas tiesiogiai priklauso nuo jų antimikrobinio aktyvumo (gebėjimo skatinti mikroorganizmų autolizę).*

Nustatyta, kad stipriausiomis antimikrobinėmis ir fagocituojančias ląsteles aktyvinančiomis savybėmis pasižymėjusiam kazeino tripsininiam hidrolizatui nebuvo būdingas apsauginis priešuždegiminis poveikis, tačiau kontaktinio hiperjautrumo reakcijos atveju 41,7 % slopino pelės pėdos edemą. Nei ūminio uždegimo, nei kontaktinio hiperjautrumo atveju nenustatyta statistiškai reikšmingų limfoidinių organų svorių ar hematologinių rodiklių pakitimų. Abiejų uždegiminių modelių atveju nustatytos padidėjusios citokinų TNF-α ir IL-10 koncentracijos.

Tiriant egzogeninių lizuojančių fermentų preparatų, lizocimo, lizosubtilino bei jų kombinacijų, poveikį somatinių ląstelių kiekiui, kurio padidėjimas rodo pieno liaukose vykstančias uždegimines reakcijas, nustatyta, kad efektyviausiai somatinių ląstelių kiekį karvių piene sumažino lizocimo ir vitaminų A, C bei E mišinys. Dešimtą dieną nuo šio mišinio skyrimo pradžios somatinių ląstelių kiekis piene sumažėjo 64,3 %.

Apibendrinant galima teigti, jog tiek maisto baltymų hidrolizatai, tiek egzogeniniai lizuojantys fermentai yra efektyvios antimikrobinės priemonės, pasižyminčios ir imunostimuliuojančiu poveikiu.

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