INFLUENCE OF β -GLUCAN (MACROGARD®) ON INNATE IMMUNITY OF CARP FRY

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Abstract

β -glucan (Macrogard®) was administrated to enhance the immunity and growth of *Cyprinus carpio* fry. The whole body homogenate of fish sampled with one week intervals during 2nd - 6th weeks post hatching was assayed for the total protein content, lysozyme activity, α -2-macroglobulin content, classical complement pathway activation, and weight gain. After the 3rd week of experimental feeding, the total protein content and the classical complement activity of fish fed β -glucan supplemented diet were higher than controls. Significantly higher lysozyme activity and α -2-macroglobulin levels were noted in group fed β -glucan at the 2nd and 3rd week of diet application. It was demonstrated that β -glucan enriched feeding increased the immunity of common carp at the earliest stages of their development.

Key words: carp, β -glucan, α -2-macroglobulin, lysozyme, classical complement pathway.

Intensive fish-breeding methods usually are accompanied with various stress factors, such as high stocking density, handling, and transportation. As most of fish farms operate in the open environment, fish are continuously exposed to pathogenic microorganisms.

It is widely agreed that immune prophylaxis is one of the promising direction of control of infectious diseases. Enhancing the immune capacity of fish during periods of strong stress may be cost effective way to decrease losses in aquaculture production.

Immune modulators, such as feed supplements and probiotics attract attention in all areas of animal production. β -glucans are long chain carbohydrates and are able to activate innate immune system (4). They were presented to have protective effect against tumour cells, viruses, bacteria, fungi, parasites, and carcinogens (15, 19). It was revealed that β -glucan acts through activation of the immune cells - macrophages, Th lymphocytes, and NK-cells (7-9).

In carp farming, the earliest developmental period is the time when fish are highly vulnerable to pathogenic microorganisms. High mortalities are commonly observed during first weeks after hatching. At this time, the immune system mainly depends on the non-specific immune defence mechanisms (27). Substances stimulating the non-specific immune defence mechanisms could improve survivorship (17). However, there is no data on the influence of β -glucan on the early stages of carp development.

The aim of the study was to investigate the effect of β -glucan (Macrogard®) on innate immunity of common carp at the earliest stages of its development.

Material and Methods

Fish. Experimental group of fish was obtained by artificial spawning in spring of 2009. The milt of two mails: W-580, UP-GO was used for simultaneous fertilisation of the eggs obtained from one female R6-229. The eggs were incubated at 20°C for 3 d. After hatching, larvae were stocked in the recirculation water system with UV treated water.

β -glucan. The commercial feed supplement Macrogard® (β -glucan), provided by Biorigin (Biorigin Scandinavia), was used in the experiment.

Experimental design. Carp progeny were fed *Artemia salina* during the first two weeks post hatching (w.p.h.). Afterwards, progeny were divided into two feeding groups - experimental and control. The experimental group was fed AllerAqua (AllerAqua, Poland) containing Macrogard® starting from the 2nd week post hatching, up to the 6th week. The daily inclusion rate of Macrogard® was 0.1% of fish body weight. The control group was fed AllerAqua alone. Water temperature, pH, and concentration of dissolved oxygen as well as total ammonia nitrogen (TAN) were monitored daily. Throughout the experimental period, water temperature was 20.2°C $\pm$ 0.5°C. The pH value was 6.85 $\pm$ 0.4. Concentration of oxygen in water was 5.54 $\pm$ 1.1 mg O₂/L. TAN was determined using phenate method (2). Concentration of ammonia nitrogen was 0.094 $\pm$ 0.21 mg N/L. The samples (whole fish) were taken on the 2nd, 3rd, 4th, 5th, and 6th w.p.h. The parameters of innate immunity were measured in the whole body homogenate. Five fish from each group

were sacrificed by exposure to 2 mL of Propiscin (2% etomidate, Inland Fisheries Institute, Poland) (15).

Homogenate. The frozen carp fry were weighed and placed in ice cold protein extraction buffer (20 mL of 1 M Tris-HCl; 0.584 g of NaCl; 0.745 g of KCl; 0.602 g of $MgCl_2$ per 1 l of distilled water). The amount of the buffer was 10 x of fish body weight (mL/mg). Next, the samples were homogenised (Pol-Eko Aparatura H500) for 2 min and Nonidet P-40 (Sigma, Poland) was added to each tube with the homogenate (0.5% of extraction buffer volume). Then, the homogenates were shaken for 15 min and centrifuged for 15 min at 4°C (10,000 x g). The supernatants were aliquoted into new tubes, frozen, and taken for analysis when required.

Total protein content was analysed following Bradford method (6). Globulin content of homogenate and albumin-globulin ratio was assayed as described by Pillemer (22). Lysozyme activity was determined as described by Parry (21). Alpha-2-macroglobulin activity was measured according to Ganrot (10). Classical complement pathway activation was assayed according to Yano (28). All assays, except the homogenisation step, were adapted to microtitre plates.

Statistical analysis. The level of significance was chosen at $P < 0.05$ (repeated measurements ANOVA). All calculations were done using MedCalc 12.2. 1.0 (MedCalc Software bvba). The results are presented as means $\pm$ standard deviation.

Results

After 3 weeks of experimental feeding, the total protein content of the whole body homogenate of carp fed β -glucan supplemented diet was significantly ($P < 0.001$) higher than in controls (4.51 ± 0.15 mg/mL and 3.98 ± 0.13 mg/mL, respectively).

Further analysis revealed that this difference was mainly caused by a higher level of globulin fraction in homogenate in the β -glucan group ($P < 0.05$) (Fig. 1).

Three weeks of experimental feeding did not affect the body weight of control group (0.655 ± 0.038 g) and group, which received β -glucan supplement (0.662 ± 0.056 g).

The measurements of lysozyme activity started at week 2 post hatching. The highest level of lysozyme activity in control group was recorded at this time of observation (5.733 μ g/mL). The activity of lysozyme significantly decreased at week 3 post hatching and remained at the same level in control group till the end of the experiment. One week of Macrogard[®] application resulted in elevation of lysozyme activity. Higher level of lysozyme in β -glucan group was maintained in the following weeks, being significantly higher at weeks 5 and 6 post hatching. (5.921 and 5.682 μ g/mL in experimental and 5.429 and 5.340 μ g/mL in control group in week 5 and 6, respectively ($P = 0.01$) (Fig. 2)

Fig. 1. Mean values ($\pm$ standard deviations) of globulin percentage after 3 weeks of experimental feeding (6 w.p.h.). $n = 6$, * $P < 0.05$

Fig. 2. Mean values ($\pm$ standard deviations) of lysozyme activity during 2-6 w.p.h. Significant differences ($P < 0.05$) between the group fed β -glucan and control group are depicted with an asterisk, while differences between initial level and time-points are displayed with a capital letter A. The number of weeks of Macrogard[®] application in experimental group is shown in parentheses

Fig. 3. Mean values ($\pm$ standard deviations) of α -2-macroglobulin activity during 2-6 w.p.h. Significant differences ($P < 0.05$) between the group fed β -glucan and control group are depicted with an asterisk, while the differences between initial level and time-points ($P < 0.01$) are displayed with a capital letter A. The number of weeks of Macrogard[®] application in experimental group is shown in parentheses

Fig. 4. Mean values ($\pm$ standard deviations) of classical complement activity during 2-6 w.p.h. Significant differences ($P<0.05$) between the group fed β -glucan and control group are depicted with an asterisk, while differences between initial level and time-points ($P<0.001$) are displayed with a capital letter A. The number of weeks of Macroguard[®] application in experimental group is shown in parentheses

The α -2-macroglobulin activity was the highest in the 2nd (5.079 units of protected trypsin-u.p.t.) and 3rd (5.194 u.p.t.) w.p.h. In the period between weeks 3 and 4 post hatching, which corresponded to the first week of β -glucan application, a significant decrease in α -2-macroglobulin activity ($P<0.05$) in both groups was observed; although, the rate of the decrease was slower in β -glucan group. Two weeks of Macroguard[®] application resulted in a significant difference between experimental and control group (4.121 u.p.t. and 3.823 u.p.t., respectively at week 5 post hatching, $P<0.05$). Continuation of β -glucan application did not maintain these differences and mean values for two groups became insignificant (Fig. 3).

The classical complement activity in two-week-old carp was not detected. In subsequent weeks, the activity of complement increased significantly ($P<0.001$) (Fig. 4). The application of β -glucan did not cause significant differences between experimental and control groups during the first two weeks of experimental feeding. However, three weeks of β -glucan application resulted in significantly ($P<0.05$) higher values of classical complement activity in the experimental group (165,072 CH50 in experimental and 123,010 CH50 in control group).

Discussion

Many authors reported stimulating effect of β -glucan on the innate immunity of fish. The mechanism by which β -glucan enhances immunity is still not elucidated completely. The present work demonstrates dynamics of changes in innate immune parameters of Common carp fry during 2-6 weeks post hatching and effect of Macroguard[®] on these parameters.

Administration of β -glucan for 2 weeks resulted in a significant increase in the total protein content in

whole body homogenate. Further analysis revealed that it was caused mainly by a significant increase in the globulin fraction. Similar results were reported for *Labeo rohita* fingerlings (19) (proteins were measured in serum), and for *Anabas testudineus* (9) (proteins were measured in homogenate). The increased production of globulins is triggered by the presence of the foreign antigen – in the analysed case it could be β -glucan.

Lysozyme is proven to be active in fish starting from the first days of development (9). It acts simultaneously with other mechanisms of the non-specific immune system, such as complement and macrophage activity (24, 25). The increase in lysozyme activity caused by β -glucan was demonstrated in fish (2, 8, 9, 19). It is widely agreed that increased lysozyme level could result in a reduced mortality of young fish facing different bacterial infections. In the study, the lysozyme level was the highest at week 2 post hatching and from the 3rd week post hatching it was maintained at a lower level throughout the whole period of observation in the control group. Application of β -glucan resulted in a significant increase in lysozyme level, comparable with the level noted at week 2 post hatching.

In *Labeo rohita*, the lysozyme C mRNA transcripts were detected from 24 h post fertilisation to 31 d, while the lysozyme G mRNA transcripts were present earlier, from unfertilised egg stage onwards (20). Studies on zebrafish (*Danio rerio*) showed that lysozyme C gene started to be visible at 20 h post fertilisation (hpf), but by 72 hpf, the lysozyme C positive cells were undetectable. Secreting cells reappeared at 6 days post fertilisation (d.p.f.) in the head kidney, where the main haematopoiesis takes place. It was also demonstrated that lysozyme C was expressed specifically in macrophages (18). On the other hand, there are data showing expression of C-type lysozyme in liver and stomach in juvenile brill (*Scophthalmus rhombus*) while G-type lysozyme in all tissues examined. It was demonstrated that bacterial infection was able to induce over-expression of G-type lysozyme only (14). In the study, it was impossible to differentiate lysozyme G from lysozyme C. A decrease in lysozyme level, noted between weeks 2 and 3 post hatching, might be connected with developmental events resembling those observed in zebrafish. It is also impossible to state, which form of lysozyme (G or C) was mostly induced by the β -glucan diet. The results obtained in whole body homogenates confirmed that probably most immune cells responsible for lysozyme secreting were formed until the 2nd week post hatching in common carp. A significant increase in lysozyme level in response to β -glucan diet suggests activation of appropriate immune cells like neutrophils, monocytes, and macrophages – main lysozyme producers (19, 30).

The similar to lysozyme pattern of changes was observed for protease-binding protein – the α -2-macroglobulin. The highest levels were noted at weeks 2 and 3 post hatching. A significant decrease between weeks 3 and 4 post hatching and relatively the same level for the following weeks: 5 and 6 post hatching was observed in control group. This decrease does not seem to be attributed to the implementation of dietary

β -glucan at week 3 post hatching and could be connected with physiological changes of developing carp (maternal effect). The α -2-macroglobulin was shown to be produced by fish embryo from about 12 h post fertilisation (12). As far as it is known, there are no data recording the effect of β -glucan on α -2-macroglobulin during the first weeks of fish development. The results show that the application of Macrogard[®] enriched diet minimised a decrease in α -2-macroglobulin between weeks 3 and 4 post hatching and maintained a significantly higher level in the experimental group during the following two weeks of observation. Again, similarly like in case of lysozyme, it could be speculated that differences between control and experimental group could be due to a higher activation of the available pool of immune cells. There are data showing that expression of C3 and α -2-macroglobulin in the liver was not affected by oral immunostimulation with yeast DNA in carp juveniles (12). C3 is produced by carp embryos from 12 h post fertilisation (12), but most of the complement proteins are synthesised as inactive precursors that function either as enzymes or as binding proteins upon activation by certain foreign substances (11, 23). This may provide an explanation, why classical complement pathway activation was observed at 2nd week post hatching.

Application of Macrogard[®] starting from the third week post hatching resulted in a significant increase in classical complement activity when compared to fish fed the control diet. An insignificant increase in this parameter caused by β -glucan was described in *Pagrus auratus* (7). Most of the available data show the effect of β -glucan on alternative complement pathway in fish (3, 18). Zhang *et al.* (29) demonstrated that *Blastomyces dermatitidis* yeast activates the human complement system, leading to deposition of opsonic complement fragments on the yeast surface. At the same time, a glucan-deficient mutant yeast delayed initiation of C3 deposition and reduced C3-binding capacity by 50%. The authors suggest that β -glucan is an initiator of complement activation and a C3 acceptor in humans. Classical complement pathway requires the presence of specific antibody for its activation. Rombout *et al.* (5) showed early (3-5 d.p.f.) activation of the recombination activating genes in the thymus of common carp, which could be referred as a starting point of T-cell dependent antibody response development. It is also possible that naturally occurring antibodies against β -glucan components might initiate complement activation.

The results demonstrate that feeding with β -glucan enriched diet enhances the immune defence parameters of juvenile carp. However, it needs to be explained, whether the similar effects will be repeated under field conditions.

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