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ACTIVITY OF PROTEASE INHIBITORS AND LYSOZYME OF HEN'S EGG WHITE DEPENDING ON FEED MODIFICATION AND EGG STORAGE

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The aim of the study was to recognise the effect of fodder modification with mineral-humine preparations, components rich in n-3 PUFA, and antioxidants (hibiscus, vit. A+E) on the activity of lysozyme, cysteine proteases inhibitor - cystatin and inhibitors of serine proteases in egg white. The experiment was carried out on 6 groups of Tetra SL laying hens: I control with standard feeding, II with the addition of 2% Humocarbovite, III with 3% KRM (fish--mineral concentrate with humocarbovite), IV with 3% KRM and hibiscus, V with 3% KRM and vit. A+E, VI with 3% rapeseed meal. Eggs, fresh and stored for 2 and 4 weeks at 15°C were investigated. Feeding affected the ability of cystatin to inhibit papain activity. For the fresh eggs the highest inhibition activity of cystatin was found for group VI, whilst the lowest for the group fed with 3% KRM and vit. A+E. After 4 weeks of storage only the residual activity of cystatin in eggs was determined. Feeding modification had a strong effect on the inhibition of the activity of ovomucoid and ovoinhibitor. The highest antitrypsin activity was noticed in eggs laid by hens fed with standard fodder or humocarbovite addition. However, feeding with 3% KRM and vit. A+E or rapeseed meal decreased the activity of ovomucoid and ovoinhibitor. After 4 weeks of storage, a high activity of inhibitors was achieved only in eggs with a high initial activity of inhibitors. Hens' fodder supplementation with the studied additives lowered the activity of lysozyme, but not more than by 10% as compared to the control group. The activity of lysozyme was at the same level in all experimental groups fed with modified fodder. The enzymatic activity of lysozyme decreased slightly during storage of eggs. It can be concluded that fodder enrichment in n-3 PUFA changed the biological activity of egg white components. Only slight effect was observed upon the addition of humcarbovite and fish mineral concentrate, while the addition of KRM with vitamins A+E significantly decreased the activity of lysozyme and inhibitors in egg white. Feeding with rapeseed meal had a positive effect on cystatin activity but lowered the activity of other components, especially during storage.

INTRODUCTION

Feeding modification of the chemical composition of an egg, especially enrichment in n-3 polyunsaturated fatty acids, vitamins and minerals, induced physiological reactions in hen's body, expressed mainly by altered levels of biologically active substances in blood. For example, a diet containing a higher amount of fats, *i.e.* plant oils, evoked changes in the egg white mass, which is connected with the stimulation of protein synthesis in oviduct. Protein synthesis is dependent on the concentration of estrogens in hen's blood serum, which is raised by the oils' addition to the birds' diet [Whitehead *et al.,* 1993]. Incorporation of fish oil, linseeds or rapeseed meal to the hen's fodder [Rudnicka *et al*., 2003], which influenced the egg yolk lipids, could also have an impact on other egg constituents like enzymes and protease inhibitors of egg whites, which protect the developing embryo. Moreover, an increase in fat content of bird's diet influenced the normal tract of cup cells and tubular glands of oviduct mucosa which product biologically active proteins.

Hen's egg white is a rich source of biologically active substances like lysozyme, cystatin, trypsin, ovomucoid and ovoinhibitor. Lysozyme is an alkaline globular protein which is characterised by a high enzymatic activity. Natural, biological functions of lysozyme are directed to the protection of a developing embryo. The lytic activity of lysozyme is connected with degradation of Gram positive (G+) bacteria cell walls, therefore it is considered to be a germicidal agent. Moreover, lysozyme inactivates viruses through tiding up their DNA and formed non-separated complexes. Lysozyme is also able to inactivate toxins outside the cell. As a strong antibacterial and antivirus substance lysozyme is widely used in food (bioprotective agent), cosmetics and pharmaceutics (natural antibiotic) industries [Cunningham *et al.,* 1991].

Cystatin has germicidal and antivirus activities, too. This protein is a very strong inhibitor towards ficin, papain and cathepsins B, H and L. Cystatin plays an important role in protein degradation control, either outside and inside the cell [Barrett, 1981], thus it found a special interest in clinical science.

Ovomucoid is isolated from the non-coagulated part of an egg white and it has an inhibitory activity towards different enzymes depending on bird's species, *i.e*. chicken ovomucoid inhibits only trypsin, duck's and turkey's egg white ovomucoid acts towards trypsin and chymotrypsin. Owoinhibitor is able to inhibit trypsin and also bacterial and fungicidal proteases [Broadway, 1997; Saxena & Tayyab, 1997]. This protein can also be an inhibitor of chymotrypsin, but its specific activity is different from that of ovomucoid's. All

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mentioned inhibitors (ovomucoid, ovoinhibitor, cystatin) possess a very high and specific activity and are highly thermostable [Acker & Ternes, 1994].

After isolation from egg white, biologically active substances are very often disposed as natural preservatives in food products, or determine the durability of food produced with the addition of egg white [Cunningham *et al.,* 1991; Kopeć & Trziszka, 1997].

The biological activity of egg white proteins is dependent on *i.e.* fodder constituents and their additives such as antibiotics (decreased the activity of lysozyme) or humine acids (extended of egg shelf life) [Rudnicka *et al*., 2003]. Modification of fatty acids composition of egg yolk lipids tends to change storage durability of eggs, especially due to an increase in polyunsaturated fatty acids content of egg yolk. Oxidation processes can be stopped by the use of an antioxidants in fodder, *i.e.* tocopherols. Oxidative changes in eggs enriched in n-3 polyunsaturated fatty acids are well recognised, however an impact of egg white enrichment with biologically active substances is a new field of knowledge.

The aim of the study was to evaluate the effect of fodder enrichment with humine preparations, components rich in n-3 PUFA, and antioxidants (hibiscus, vit. A+E) on the activity of lysozyme, cystatin and inhibitors of serine proteases in egg white.

MATERIALS AND METHODS

The study was carried out on eggs collected from the inter-breed hybrids of laying type hens Tetra SL kept in battery cages. The experiment was conducted on 6 groups of hens which were fed: with standard fodder (energy 2700 kcal/kg, 17% protein) - group I control, group II with the addition of 2% of humocarbovite, III with 3% KRM added (fish and minerals concentrate with humocarbovite), IV with the addition of 3% KRM + hibiscus, V with 3% KRM + vit. A+E, VI with 3% of rapeseed meal. Humocarbovite is a mineral–huminic additive containing 30% of huminic acids and 23% ash (1.7% Ca, 0.6% P, 800 mg/kg Fe, 440 mg/kg Mg, 140 mg/kg Mn and 17 mg/kg Cu). KRM is a mixture of humocarbovite and fish oil in the proportion of 2:1. Fish oil contained 18% of n-3 PUFA. As antioxidants dried petals of hibiscus (*Hibiscus rosa-sinensis*) containing about 500 mg/100g anthocyanins in the amount of 0.01% per kg fodder or a mixture of vitamin A (10000 units/kg) and E (20 mg/kg) were applied. As a source of polyunsaturated fatty acids also rapeseed meal containing 6% n-3 PUFA was used. Eggs were analysed immediately after collection and after 2 and 4 weeks of storage at a temperature of 15°C. Activities of biologically active substances present in egg white, *i.e.* lysozyme, cystatin and serine proteases inhibitors, were analysed on 32 eggs from each experimental groups.

The reaction of trypsin with the synthetic substrate BApNA (N-benzoilo-DL arginino p-nitroanilid) was used in order to determine antitrypsin activity of egg white [Broadway, 1997]. During the enzymatic reaction p-nitroanilin (yellow) is released from the substrate and maximum absorption is measured at a wavelength of 412 nm. Egg white samples were incubated with an appropriate amount of added inhibitor in a buffer containing Ca^{++} at a temperature of 37°C. The ability of egg white to inhibit trypsin activity was analysed by lowering the absorbance at 412 nm compared to control samples (without added inhibitor).

Lysozyme activity was analysed spectrophotometrically. The method is based on the measurement of absorbance changes of the solution of *Micrococcus lysodeicticus* bacteria during the reaction of enzyme with the bacteria cell. Measurements of the samples were taken at a stable temperature of 25°C at a wavelength of λ=450 nm every 60 sec for 6 min of the reaction.

The inhibitory activity of cystatin against papain was analysed according to Nishida *et al.* [1984] and Siewiński [1991]. The method is based on colorimetrical measurements of the amount of products released from the substrate BANA (N-benzoil-DL-arginyl-β-naphtylamide hydrochloride) by the action of cysteine protease – papain after 20 min of incubation at a temperature of 37°C. The reaction was stopped by adding of 9,10-dimethyl-1,2-benzoanthracene in acetic acid. Absorbance of samples was measured at a wavelength of λ=450 nm. One unit of the inhibitory activity of cystatin corresponds with one unit of the enzymatic activity of papain, which is the amount of enzyme capable of hydrolysig 1.0 mmol of substrate per one minute under standard conditions (37°C).

All the results were then analysed statistically using Statistica 6.0 programme with one-way analysis of variance at the significance level of p=0.05.

RESULTS AND DISCUSSION

The study was conducted on egg white collected from hen's fed with the fodder enriched with mineral-humine preparations, n-3 polyunsaturated fatty acids and antioxidants (hibiscus, vitamins A+E). Analyses of the activity of biologically active substances were carried out on hen's eggs fresh and stored at a temperature of +15°C, which reflected much better the conditions of Polish egg retail market than the classical refrigerating chain (+4°C).

Feeding modifications applied in the experiment had a significant impact on the inhibitory activity of cystatin. Differences in cystatin activity analysed in fresh egg white amounted to approximately 20% within studied groups (Table 1). A high cystatin activity was measured for egg whites from the control group and block of hens fed with diet enriched with rapeseed meal (approximately 12 units/5 mg protein). A slightly lower activity of cystatin was analysed for eggs collected from birds fed with fodder with the humocarbovite addition and with fish oils (approximately 10 units/5 mg protein), whereas the lowest inhibitory activity of cysteine proteases was measured for the group fed with a diet enriched with 3% of KRM and vitamins A+E (below 10 units/5 mg protein) (Table 1). The activity of cystatin obtained for the control group in the experiment was comparable to those reported by Świerczewska *et al*. [2005]. Thus, it can be concluded that hens' feeding modification by enrichment with fish-mineral concentrate decreased the activity of egg white cystatin. The results of the study proved that the ability of egg white cystatin to inhibit papain was

TABLE 1. Cystatin activity in fresh and stored egg white in relation to hen's feed modification.

Variants	Cystatin activity (units/5 mg protein)		
	fresh	stored for 2 weeks	stored for 4 weeks
I - control	$12.10^{a} \pm 0.98$	$11.22^{b} \pm 0.49$	$1.38^{i} \pm 0.09$
II – 2% humocarbovite	$10.43^{c} \pm 0.94$	$9.40^{e} \pm 0.55$	$0.57^{j} \pm 0.05$
III – 3% KRM	$11.75^{ab} \pm 0.42$	$11.23^{b} \pm 0.28$	$0.73^{j} \pm 0.05$
IV – 3% KRM + hibiscus	$10.07^{cd} \pm 0.75$	$6.22^{f} \pm 0.39$	$1.34^{i} \pm 0.10$
V – 3% KRM + vit. A+E	$9.68^{de} \pm 0.99$	$4.19^{g} \pm 0.22$	$0.74^{j} \pm 0.02$
VI – 3% rapeseed meal	$12.30^{a} \pm 0.82$	$3.34^{h} \pm 0.76$	$1,32^{i} \pm 0.11$

a, b, c, d, e, f, g, h, i, j – the same letter in indices of means shows no significant differences at $p \leq 0.05$

TABLE 2. Antitrypsin and antichymotrypsin inhibitory activity in fresh and stored egg white in relation to hen's feed modification.

Variants	Antitrypsin and antichymotrypsin inhibitory activity (units/0.1 mg protein)		
	fresh	stored for 2 weeks	stored for 4 weeks
I - control	$23.31^{b} \pm 0.75$	$13.69^{d} \pm 0.70$	$13.08^{e} \pm 0.18$
II – 2% humocarbovite	$26.32^{a} \pm 0.46$	$11.69^{f} \pm 0.73$	$10.20^{g} \pm 0.34$
III – 3% KRM	$11.12^{f} \pm 0.77$	$10.03^{g} \pm 0.77$	$4.79^{k} \pm 0.23$
IV – 3% KRM + hibiscus	$16.45^{c} \pm 0.93$	$11.50^{f} \pm 1.02$	$2.52^{m} \pm 0.20$
V – 3% KRM + vit. A+E	$9.03^{h} \pm 0.42$	$5.68^{j} \pm 0.73$	$3.67^{l} \pm 0.15$
VI – 3% rapeseed meal	$8.63^{h} \pm 0.64$	$7.89^{i} \pm 0.32$	$3.23^{l} \pm 0.19$

a, b, c, d, e, f, g, h, i, j, k, l, m – the same letter in indices of means shows no significant differences at $p \leq 0.05$

significantly dependent not only on hen's age [Trziszka *et al*., 2004b; Świerczewska *et al*., 2005], genetics and breeding system [Świerczewska *et al*., 2003b], but also on fodder modifications and feeding system.

Eggs collected from hens fed with rapeseed meal-enriched diet were characterised by poor storage stability. The activity of cystatin analysed in egg white from this experimental block decreased significantly after 2 weeks of storage at a temperature of 15°C. There were no significant changes in cystatin activity in egg white collected from hens fed with standard fodder and with humocarbovite or fish oil addition, but without antioxidants (Table 1). Storage of eggs up to 4 weeks caused a significant decrease of cystatin activity in all experimental groups. The results of the study supported the thesis that protective mechanisms of developing embryo expressed by cysteine protease inhibitors are reduced during storage of eggs, which was reported first by Trziszka *et al*. [2004a].

Applied in the study differences in hen's feeding influenced, much more than cystatin activity, the activity of serine proteases inhibitors, which was expressed as an ability of ovomucoid and ovoinhibitor to inhibit the activity of bovine trypsin (Table 2). The highest antitrypsin activity was analysed in egg white collected from hens fed with fodder with humocarbovite addition and also in eggs from the control group of birds (more than 20 units/0.1 mg protein). The results obtained were comparable to data published by Świerczewska *et al*. [2005]. Incorporation of either fish oil with concentrate of mineral substances or rapeseed meal to the hen's diet significantly decreased (from 40% to 70%) the activity of serine protease inhibitors in egg white (Table 2).

After 2 weeks of storage at a temperature of 15°C, the activity of trypsin inhibitors decreased in all analysed egg white. Extended storage (up to 4 weeks) tend to further decrease the activity of ovoinhibitor and ovomucoid in eggs from groups III, IV, V and VI (Table 2), whereas in eggs from groups I and II, which were characterised by a high initial level of serine protease activity, the inhibitory activity changes were much lower. Results collected in the study showed that hen's feeding enrichment with n-3 polyunsaturated fatty acids had a negative effect on the activity of biologically active substances in egg white. Moreover, such fodder modifications caused acceleration of the biologically active substances activity loss during storage of eggs.

It is a well known fact that growth stimulators used in poultry breeding had a positive effect on egg laying, egg weight, *etc.* [Bessei, 1994; Świerczewska *et al*, 2003a], but there is still a lack of knowledge concerning their impact on the activity of biologically active substances in egg white such as lysozyme. Feeding modifications applied in the experiment, *i.e.* fodder enrichment with lipids and minerals, decreased the activity of lysozyme in relation to control group of birds (standard diet) (Table 3). Implementation of fish-mineral concentrate, rapeseed meal, humocarbovite or antioxidative substances to the hen's feeding lowered the enzymatic activity of egg white lysozyme, however the observed differences were not higher than 10% for each experimental groups. The average activity of lysozyme amounted to 6 700 units/5 mg protein.

TABLE 3. Lysozyme activity in fresh and stored egg white in relation to hen's feed modification.

Variants	Lysozyme activity (units/5 mg protein)		
	fresh	stored for 2 weeks	stored for 4 weeks
I - control	$7415^{a} \pm 110$	$7386^{a} \pm 265$	$7309^{a} \pm 132$
II – 2% humocarbovite	$6694^{bcd} \pm 263$	$6648^{cde} \pm 80$	$6292^{gh} \pm 43$
III – 3% KRM	$6881^{b} \pm 76$	$6389^{fgh} \pm 309$	$6309^{gh} \pm 217$
IV – 3% KRM + hibiscus	$6534^{def} \pm 163$	$6496^{defg} \pm 183$	$6404^{fgh} \pm 243$
V – 3% KRM + vit. A+E	$6752^{bc} \pm 138$	$6229^{hi} \pm 385$	$6055^{i} \pm 306$
VI – 3% rapeseed meal	$6638^{cde} \pm 304$	$6458^{efg} \pm 225$	$6415^{fgh} \pm 81$

a, b, c, d, e, f, g, h, i – the same letter in indices of means shows no significant differences at $p \leq 0.05$

For the control group, a high lytic activity of lysozyme was stable during the whole storage period (up to 4 weeks at the temperature of 15°C), whilst in eggs enriched with fish-mineral concentrate (groups III and V) significantly lower activity of lysozyme was demonstrated (Table 3). The highest decrease in lysozyme activity during storage of eggs was measured for group V, *i.e.* fed with fodder enriched with 3% of KRM and vitamins A+E. It can be concluded that feeding modifications had much lower effect on the activity of lysozyme than hen's age or hen's origin [Świerczewska *et al.*, 2005].

CONCLUSIONS

1. Modification of hen's diet through the addition of a mineral–humine preparation (humocarbovite) or its concentrate with fish oil to the fodder had only a minor effect on the activity of biologically active substances present in fresh and stored eggs (a decrease was observed only in antitrypsin activity).
2. Incorporation of rapeseed meal to the hen's diet increased the activity of cystatin, however decreased the activity of other biologically active substances in egg white, especially during storage of eggs.
3. Vitamins (A+E) addition together with n-3 polyunsaturated fatty acids decreased the activity of biologically active substances in fresh eggs. Moreover, this feeding modification reduced developing embryo protective mechanisms during storage.
4. Storage of eggs up to 4 weeks at a temperature of 15°C decreased the activity of serine proteases inhibitors even by 50%, the activity of lysozyme by approximately 10%, and the activity of cystatin was analysed only at the trace level.

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AKTYWNOŚĆ INHIBITORÓW PROTEAZ ORAZ LIZOZYMU W BIAŁKU JAJA KURZEGO W ZALEŻNOŚCI OD MODYFIKACJI ŻYWIENIA I PRZECHOWYWANIA JAJ

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Celem badań było określenie wpływu żywienia kur paszą z dodatkiem preparatów mineralno – huminowych, wzbogaconą w kwasy wielonienasycone n-3 oraz antyoksydanty (hibiskus i wit A + E) na aktywność lizozymu, cystatyny oraz owomukoidu i owoinhibitora w białku jaja. Eksperyment przeprowadzono na 6 grupach niosek żywionych: standardowo (I), z dodatkiem 2% Humokarbowitu (II), z 3% KRM (koncentrat rybno-mineralny z humokarbowitem) (III), z 3% KRM + hibiskus (IV), z 3% KRM + wit. A+E (V), z dodatkiem 3% śruty rzepakowej (VI). Badano jaja świeże oraz przechowywane przez 2 i 4 tygodnie w temp. 15°C. Najwyższą aktywność cystatyny w białku świeżym, oznaczono dla grupy VI, najniższą natomiast dla grupy z KRM i wit. A+E. Po 4 tygodniowym przechowywaniu oznaczono jedynie resztkową aktywność cystatyny. Zróżnicowane żywienie w większym stopniu niż na aktywność cystatyny wpłynęło na zdolność inhibowania trypsyny przez owomukoid i owoinhibitor. Najwyższą aktywność antytrypsynową oznaczono w białku jaj kur żywionych paszą z dodatkiem humokarbowitu oraz grupie kontrolnej; wprowadzenie do paszy KRM z witaminami A+E oraz rzepaku spowodowało obniżenie aktywności inhibitorów trypsyny. Po 4 tygodniach wysoką aktywność zachowały tylko jaja o wysokim wyjściowym poziomie aktywności inhibitorów. Zarówno zmiany w żywieniu kur, jak i przechowywanie jaj, prowadziły do nieznacznego obniżenia aktywności lizozymu (ok. 10%) w stosunku do grupy kontrolnej. Wzbogacenie paszy niosek w dodatki podwyższające zawartość kwasów n-3 powoduje zmiany aktywności substancji biologicznie aktywnych w białku jaja. W najmniejszym stopniu na te aktywności w jajach świeżych oraz podczas przechowywania wpływa dodatek humokarbowitu oraz jego koncentratu z olejem rybim, jednak zastosowanie tego dodatku wraz z witaminami A + E obniża aktywność lizozymu i inhibitorów. Wprowadzenie rzepaku jako źródła kwasów n-3 wpływa korzystnie na wzrost aktywności cystatyny, obniża jednak aktywność innych składników podczas przechowywania jaj.