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The effect of protein-xanthophyll concentrate (PX) from alfalfa (*Medicago sativa* L.) on the activity of selected enzymes in the liver cells of fattening pigs

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The available Polish and foreign literature contains few reports on the activity of indicator enzymes, especially marker enzymes, which characterize the work of specific cell organelles. Information on enzyme changes induced by experimental dietary factors is also scarce. We postulated that protein-xanthophyll PX alfalfa concentrate would have a positive effect on metabolism in fattening pigs, resulting in improved performance parameters. Therefore the aim of the study was to test the possibility of using enzymes that have not previously been used to assess the biochemical processes taking place following administration of PX. We evaluated the activity of enzymes associated with key biological transformations taking place in the cell, as this makes it possible to assess whether these processes are proceeding normally and to evaluate the response of the body to external factors. The experiment was conducted on 288 finishers (gilts and barrows), all of which were crossbreds (Polish Large White x Neckar), divided into 4 groups according to the dosage and duration of administration of protein-xanthophyll PX concentrate from alfalfa *(Medicago sativa* L.). The alfalfa concentrate was introduced to the compound feed in place of soybean extraction meal. We analysed the activity of selected marker enzymes: lactate dehydrogenase, malate dehydrogenase, succinate hydrolase, and glucose-6-phosphate hydrolase. We found a decrease in the activity of the enzymes in the liver cells of the pigs receiving the PX concentrate as compared to the control, which suggests that the product had a favourable effect on their metabolism. The results confirm the benefit of using protein-xanthophyll PX in pig feed, particularly at a continuous dose of 3%.

KEY WORDS: pigs / feed additives / alfalfa / indicator enzymes

In the European Union, under regulation 1831/2003 [35], the use of antibiotic growth promoters in raising livestock has been prohibited since 1 January 2006. This has led to

attempts to find alternative, safe sources of additives for compound feed. Supplements are administered in order to achieve high performance and good health in animals and to ensure that the food produced is of the highest quality. Feed additives include probiotics, feed enzymes, oligosaccharides, organic acids, natural minerals and herbs, which are the basis of phytobiotics, due to properties resulting from the active substances contained in them [15, 40]. An example of phytobiotics is herbs, used as an alternative to antibiotic growth promoters. Herbs contain secondary metabolites, which include alkaloids, glycosides, tannins, saponins, essential oils, terpenes, flavonoids, plant mucilages, pectins, organic acids, vitamins and mineral salts. These compounds regulate digestive functions and improve the motility and secretory functions of the gastrointestinal tract [15]. Furthermore, they support metabolism in animals (*Polygonum aviculare*) and have antibacterial and anti-inflammatory effects (garlic, onion and sage) and anti-diarrheal effects (*Aegle marmelos*). Due to the properties of secondary metabolites present in feed additives, improvement is observed in the performance parameters of livestock, their health, and meat quality [42].

Research has shown that enrichment of complete compound feed with plant components can aid regulation of digestive functions in animals (turmeric, cayenne pepper, ginger, caraway and fenugreek), improve immune activity (ginger, ginseng root, linden blossom, horsetail, and the leaves of narrow-leaf plantain and nettle), reduce susceptibility to stress (black pepper, coriander, liquorice, rosemary, ginger, caraway and cloves) and reduce the risk of oxidative stress (turmeric, vanilla, thyme, sesame and sage) [23]. Valuable plant additives also include alfalfa (*Medicago* L.). Alfalfa provides the largest amount of protein per hectare of farmland among fodder crops (e.g. rapeseed, maize, soybean and wheat) [26]. Its chemical composition is dominated by hypocholesterolaemic and antimicrobial saponin glycosides, sterols with antioxidant activity, coumestans, such as biochanin A, daidzein, genistein and coumestrol, and isoflavonoids with anti-tumour and anti-inflammatory potential [44]. This plant is also a valuable source of vitamins, provitamins and amino acids [13, 26]. Among minerals, it contains calcium, zinc, phosphorus, copper, iron, magnesium, potassium and silicon, and among vitamins, B vitamins B_1, B_2, B_3, B_5 and B_6, as well as A, C, D, E, K and beta-carotene. Alfalfa contains both proteinogenic amino acids such as isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, and non-proteinogenic amino acids, such as L-canavanine [13].

In animal diets, this plant may be used as green forage, hay or silage, or in chopped and dried form, but in the case of pigs, which are monogastric animals, not all of these types can be used [5]. It is most commonly given to pigs as green forage or in chopped and dried form, which is considered a valuable substitute for cereal bran, broad beans or peas. An interesting alternative is concentrates: protein-xanthophyll (PX) obtained from concentrated juice and EFL extract obtained from dried leaves. PX concentrate is a high-protein feed for animals, while EFL is intended for people

[2]. Alfalfa is also successfully used in concentrate form to feed carp, cattle, poultry, lambs and pigs [8, 34].

Protein-xanthophyll (PX) concentrate has a positive effect on fattening performance in pigs. Grela et al. [16] tested the effect of 2% dietary supplementation with PX on weight gains in fattening pigs, and achieved daily gains of 771 g in the control animals and 846 g in those receiving PX. Furthermore, feed conversion was reduced by 0.29 kg/kg body weight gain as compared to the control. In a study by Czech and Semeniuk [6], PX was shown to increase the lymphocyte count and decrease the neutrocyte count, which is a sign of improved immune function as a result of administration of this supplement.

Enzyme activity tests are used to monitor animal health, but are now increasingly used to evaluate the effects of other factors on the organism, such as stress, xenobiotics of various origins, or additives used to improve growth performance. Plant-based preparations can directly or indirectly cause changes in enzyme activity in the body. Research based on analysis of enzyme activity is quite common, but experiments using alfalfa products are rare. Comparison of the results with reference data is difficult because the literature lacks reports of analysis of certain enzyme parameters in livestock. Indicator enzymes are characterized by significant organ specificity, and changes in their activity are usually associated with a specific disease entity. The activity of these biocatalysts in normal serum is lower than that observed within cells. In the case of organ damage, the enzyme leaks from the cell, resulting in an increased amount of the protein in the serum [20].

The experiment evaluated the effect of PX on the activity of selected marker enzymes: glucose-6-phosphate hydrolase, succinate dehydrogenase, lactate dehydrogenase and malate dehydrogenase. These enzymes were chosen because they are helpful in assessing the course of key biochemical processes and the function of cellular organelles involved in these processes. Changes in their activity in the cell will therefore indicate the intensity of the process taking place and reflect the body's response to external factors.

Glucose-6-phosphate hydrolase (G6PC) is an indicator enzyme present in the smooth endoplasmic reticulum. It catalyses dephosphorylation of glucose-6-phosphate, which is generated by conversion of glycogen. The removal of the phosphate residue produces glucose, which is a source of energy for living organisms. This enzymatic protein is responsible for the transport of this sugar and is therefore commonly found in liver and kidney tissues, which are responsible for systemic metabolism [14, 20]. G6PC is also responsible for detoxification, and glucose released into the bloodstream is an incompetent inhibitor of this enzyme, whose activity increases with age and then decreases in the final stage of fattening. This can be explained by the ageing of the endoplasmic reticulum due to lipid peroxidation and increased free radical synthesis [30]. An increase in G6PC

activity can be explained by intensification of the gluconeogenesis process, e.g. in the case of stress or overcrowding [9, 38].

Dehydrogenase succinate (SDH) is a flavoprotein involved in the Krebs cycle. By binding to FAD and three non-haem iron atoms, it forms an enzyme complex which catalyses the conversion of succinate to fumarate and the transfer of electrons in the respiratory chain [4]. Research on succinate dehydrogenase activity in disease conditions has shown that this enzyme also has a significant role in redox reactions [37].

Malate dehydrogenase (MDH) is an enzyme catalysing the oxidation reaction of L-malate into oxaloacetate in the Krebs cycle, owing to which it can then attach another acetyl residue [27]. In addition, MDH transfers reducing equivalents to the nicotinamide adenine dinucleotide (NAD^+) to produce its reduced form NADH. Some authors claim that the activity of this enzyme is increased by oxidative stress, so analysis of MDH activity can be used to assess the severity of stress in the body. It is possible that stress is an inducer of this enzyme, which is linked to intensification of the Krebs cycle due to increased energy requirements during stress [29, 30].

Lactate dehydrogenase (LDH) catalyses reversible conversion of pyruvate to lactate. This means that the direction of the transformation depends on the ratio of pyruvate to lactate and NADH to NAD^+. Lactate dehydrogenase has significant diagnostic value because it is used as a marker of skeletal muscle damage. An increase in the activity of this protein in the blood indicates damage to muscle cell structures, but may also be a response to physical exertion or disease [33]. LDH levels are affected by the sex and age of the animals and the duration and intensity of physical exertion [18]. In the case of conditions where tissue necrosis occurs (damage to the myocardium, kidneys, skeletal muscles, liver and lungs), an increase in lactate dehydrogenase activity is observed [30].

It was postulated that PX concentrate would have a positive effect on cell metabolism, which would result in improved performance indicators in fattening pigs. The aim of the study was to test the possibility of evaluating selected biochemical processes taking place following administration of PX concentrate using enzymes that have not previously been used.

Material and methods

The material for the experiment consisted of 288 crossbred (Polish Large White x Neckar) fattening pigs with an initial body weight of 20.0 ±0.5 kg. The pigs were divided into 4 groups, differentiated by the dosage and duration of administration of PX concentrate. Each group comprised 12 pigs – 6 barrows and 6 gilts in 6 replications. All animals received a standard compound feed (grower and finisher) adjusted to the age group. The pigs were kept in a piggery in a shallow litter system in standard environmental conditions for the species, as proposed by Rokicki and Kolbuszowski [36]. The animals rece-

ived ad libitum standard compound feed from automatic feeders and had constant access to fresh water from cup drinkers. The temperature, relative humidity and air movement were maintained by automatic mechanical ventilation. Rearing conditions were identical for all experimental groups.

The control group (K) consisted of animals receiving only the standard compound feed. Pigs from experimental group D1 received protein-xanthophyll (PX) concentrate in the amount of 1.5%. Pigs from groups D2 and D3 received the same supplement in the amount of 3%. In group D2, the supplement was administered periodically – daily for 14 days, followed by a 14-day break. The pigs in group D3 received feed with the supplement continuously. The experimental design is shown in Table 1, and the composition of the compound feed is presented in Table 2.

Homogenates were prepared from the liver samples taken during dissection, in which spectrophotometric measurements of the activity of selected indicator enzymes were made. Lactate dehydrogenase activity (LDH, EC 1.1.1.27) is shown by the rate of disappearance of NADH absorbance. This technique, called the Wróblewski method, consists in monitoring the conversion of pyruvate to lactate at 25°C and 340 nm [17]. Succinate dehydrogenase activity (SDH, EC 1.3.99.1) was determined on the basis of reduction of 2,6-dichlorophenolindophenol at a wavelength of 580 nm [3]. The method described by Arfman et al. [1] was used to determine the activity of malate dehydrogenase (MDH, EC 1.1.1.37). Glucose-6-phosphate hydrolase activity (G6PC, EC 3.1.3.9) was tested by

Table 1
Experimental design

Specification	Groups			
	K	D1	D2	D3
Number of pigs	12	12	12	12
Amount of PX in feed (%)	0.0	1.5	3.0	3.0
System of feed supplementation with PX product	no PX supplementation	continuous	periodic*	continuous

K – pigs receiving standard compound feed (control)
D1 – pigs receiving standard compound feed supplemented with PX in the amount of 1.5%
D2 – pigs receiving standard compound feed supplemented with PX in the amount of 3% periodically
D3 – pigs receiving standard compound feed supplemented with PX in the amount of 3% continuously
*PX supplementation for 14 days, no PX supplementation for next 14 days

Table 2
Composition (%) of feed used in each fattening period

Components	Fattening period							
	grower (20-65 kg)				finisher (65-115 kg)			
	K	D1	D2	D3	K	D1	D2	D3
Barley	50.0	50.0	50.0	50.0	54.5	52.5	52.5	52.5
Wheat	37.0	35.5	34.0	34.0	35.0	35.5	34.0	34.0
MPU[1]	12.5	12.5	12.5	12.5	10.0	10.0	10.0	10.0
Preservative[2]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
PX	0.0	1.5	3.0	3.0	0.0	1.5	3.0	3.0

K – pigs receiving standard compound feed (control)
D1 – pigs receiving standard compound feed supplemented with PX in the amount of 1.5%
D2 – pigs receiving standard compound feed supplemented with PX in the amount of 3% periodically
D3 – pigs receiving standard compound feed supplemented with PX in the amount of 3% continuously
[1]MPU – 38% protein; 2.5% fat; 4.7% lysine; 0.9% methionine; 1.2% methionine+cysteine; 1.9% threonine; 0.45% tryptophan; 45.5 g/kg Ca; 12.0 g/kg P; 13.00 g/kg Na; 100.0 mg/kg Cu; 667.00 mg/kg Fe; 667.00 mg/kg Zn; 53.300 IU vit. A; 13.300 IU vit. D_3; 266.0 mg/kg vit. E; 7.0 mg/kg riboflavin; 4.0 mg/kg vit. B_6; 0.1 mg/kg vit. B_{12}; 40.0 mg/kg nicotinic acid; 27.0 mg/kg pantothenic acid; 9.33 MJ ME
[2]Preservative – mixture of acids: phosphoric, formic, propionic, lactic, citric, acetic and benzoic

Swanson's method, involving measurement of inorganic phosphorus released from glucose-6-phosphate [41].

The results for enzyme activity were presented in international units (U/l) per mg of protein, whose concentration was determined by the Lowry method [25]. Statistical analysis of the data was then was then performed using STATISTICA ver. 13.0 PL. Significance of differences between means was determined by the Tukey multiple-comparison test following interpretation of the results of one-way analysis of variance, at a significance level of $p \leq 0.05$.

Results and discussion

The results obtained indicate that the use of the protein-xanthophyll (PX) concentrate from alfalfa in the diet of the pigs had a significant effect on malate and lactate dehydro-

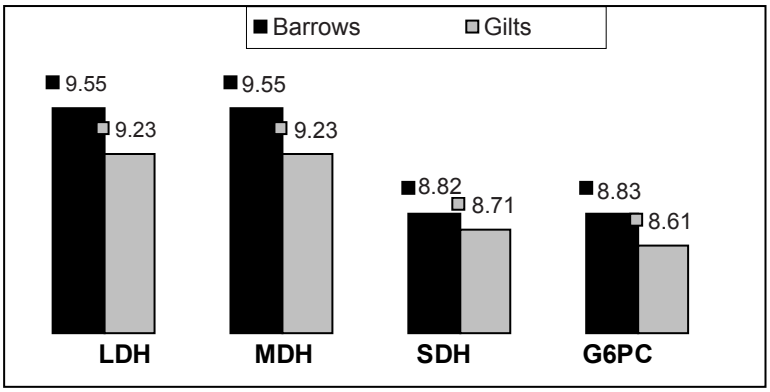

Fig. Activity of indicator enzymes in liver cells of barrows and gilts

genase and glucose-6-phosphate hydrolase. In the case of succinate dehydrogenase, no significant changes in activity were induced by the experimental factor. Enzyme activity also varied depending on the sex of the animals (Fig.), but these values did not differ from the reference standards proposed by other authors [7, 19].

Glucose-6-phosphate hydrolase activity (G6PC) ranged from 29.45 to 38.86 U/l, with slightly higher values observed for barrows than for gilts (Figs. 3 and 4). However, the differences observed were not statistically different. The results for G6PC activity in group D1 showed similar values to the control group. In the D2 group, periodic administration of PX in the amount of 3% of the compound feed led to significantly ($p \leq 0.05$) lower activity of this enzyme, by 7.2% in the males and 11.44% in the females, than in the pigs fed the standard compound feed. Continuous administration of PX concentrate in the amount of 3% resulted in a decrease in glucose-6-phosphate hydrolase activity of 7.77% in the barrows and nearly 11% in the gilts. The lower enzyme activity in the groups receiving PX can be considered a positive phenomenon, given the role of this protein in lipid peroxidation and free radical synthesis [31, 32]. Similar results were obtained in studies on turkeys [12] and chickens [28]. Furthermore, lower G6PC activity may indicate the absence of intensification of gluconeogenesis, as observed during stress, as well as better availability of the energy contained in glycogen [20]. The reverse response, involving a decrease in the activity of the enzyme, is probably age-related [39].

The activity of malate dehydrogenase (MDH) in the liver tissue was at a level of 80.62-99.72 U/l (Tables 3 and 4). In group D2, the decrease observed in activity was smaller than in group D3. Periodic supplementation with 3% PX concentrate resulted in a decrease in

Table 3
Activity of indicator enzymes in liver cells of barrows ($\bar{x}$ ±SD)

Enzyme (U/l)	Groups				
	K	D1	D2	D3	p-value
LDH	9.55^{a} ±0.8	9.55^{a} ±2.52	8.82^{b} ±0.94	8.83^{b} ±3.01	0.031
MDH	99.72^{a} ±1.06	96.6^{a} ±3.81	95.94^{b} ±0.53	80.62^{c} ±3.0	0.001
SDH	6.71 ±0.94	6.81 ±3.87	6.84 ±0.77	6.57 ±2.38	0.93
G6PC	38.86^{a} ±0.76	36.37^{ab} ±4.92	36.06^{b} ±0.8	35.84^{c} ±2.08	0.026

K – pigs receiving standard compound feed (control)
D1 – pigs receiving standard compound feed supplemented with PX in the amount of 1.5%
D2 – pigs receiving standard compound feed supplemented with PX in the amount of 3% periodically
D3 – pigs receiving standard compound feed supplemented with PX in the amount of 3% continuously
a, b, c – statistically significant results are designated with different letters

Table 4
Activity of indicator enzymes in liver cells of gilts ($\bar{x}$ ±SD)

Enzyme (U/l)	Groups				
	K	D1	D2	D3	p-value
LDH	9.23^{a} ±0.82	9.23^{a} ±0.33	8.71^{ab} ± 0.76	8.61^{b} ±0.85	0.031
MDH	92.64^{a} ±3.87	93.56^{a} ±3.42	90.69^{b} ±6.61	82.63^{c} ±7.61	0.013
SDH	6.73 ±1.12	6.65 ±0.49	6.63 ±0.78	6.86 ±0.42	0.95
G6PC	33.47^{a} ±3.89	29.45^{b} ±0.86	29.64^{b} ±1.93	29.82^{b} ±1.74	0.02

K – pigs receiving standard compound feed (control)
D1 – pigs receiving standard compound feed supplemented with PX in the amount of 1.5%
D2 – pigs receiving standard compound feed supplemented with PX in the amount of 3% periodically
D3 – pigs receiving standard compound feed supplemented with PX in the amount of 3% continuously
a, b, c – statistically significant results are designated with different letters

enzyme activity of almost 4% in the barrows and 2.1% in the gilts as compared to the control. Similarly, in group D3, these values differed statistically ($p \leq 0.05$) by 19% and nearly 11%. An increase in MDH activity may suggest stress in animals, which is most likely an

inducer of this enzyme, as confirmed by research by Neeraj et al. [29] on fish. This may be explained by an increased demand for energy at that time, and thus by intensification of transformations taking place during the Krebs cycle.

Lactic dehydrogenase (LDH) activity reached values of 8.82-9.55 U/l in the barrows and 8.61-9.23 U/l in the gilts (Tables 3 and 4). The use of 3% supplementation in the barrows resulted in lower activity of the enzyme than in the control, by 7.77% and 7.64% in groups D2 and D3, respectively. In gilts this dependence was observed only in group D3, where the value was significantly ($p \leq 0.05$) lower, by 6.72% as compared to the control. LDH activity in gilts did not differ from the norms reported by Winnicka [43], but for the boars in the K and D1 groups the values deviated slightly from the established norms. Kędzierski and Przychodzeń [18] suggest that the dynamics of this protein may also depend on the age of the animals. Experiments conducted by Krauze and Grela [24] on turkeys indicated a significant effect of protein-xanthophyll PX on lactate dehydrogenase activity. In that study biochemical parameters were analysed in the blood of turkeys fed PX in the amount of 1.5% and 3% of feed. The activity of the enzyme was significantly reduced in the case of a 3% share of the additive, which is considered to be beneficial because the reverse situation (an increase in enzyme activity) could indicate the occurrence of disease [21, 22]. This enzyme occurs naturally in the cell cytoplasm and it enters the blood following cell death and in the case of increased cell membrane permeability [30].

No statistically significant differences were observed in the dynamics of the enzyme SDH between individuals belonging to groups receiving different levels of PX. The change in activity between the control and the remaining groups remained at 2%. Succinate dehydrogenase activity ranged from 6.57-6.84 U/l in the case of barrows and 6.63-6.86 U/l in the gilts. Krauze and Grela [24] showed that the use of PX at different doses in 8-week-old turkeys of the same sex does not affect SDH activity. The lack of change in the activity of this enzyme following the use of PX is a beneficial phenomenon. Mitochondrial damage is thought to increase the activity of this enzyme. It is therefore concluded that a low level of succinate dehydrogenase indicates that this organelle is functioning properly [10, 11].

A lack of increase in the activity of the enzymes analysed should therefore be considered a beneficial phenomenon, as such a change would accompany an increased metabolic rate, which is undesirable in terms of the needs of the organism and the consequences of overproduction of various metabolites, such as free radicals.

It can be concluded from the study that supplementation with the protein-xanthophyll (PX) preparation from alfalfa has a beneficial effect on the activity of the enzymes analysed, especially glucose-6-phosphate hydrolase and lactate and malate dehydrogenase. The reduced activity of the enzymes, in particular in the group of pigs fed a diet

with a 3% addition of protein-xanthophyll (PX) concentrate, administered continuously, improves cell metabolism. The results show that PX can be used as an additive to improve the health and growth performance of pigs.

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