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The activity of acid phosphatase and β-N-acetyl-hexosaminidase in raw whole milk of cows as affected by feeding season (autumn/winter *vs*. spring/summer)

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An attempt was made at identifying the activity of acid phosphatase (AcP) and β-N-acetylhexosaminidase (Hex) in the whole raw milk of cows maintained on ecological farm and over the autumn/winter and spring/summer seasons (AW and SS season, respectively) fed with typical feeding rations. Both enzymes play a crucial role in animals' metabolism, affect the technological properties of milk and are indicators of homeostasis in animal tissues, including *mastitis*.
The effect of AW season was estimated on 23 while that of SS on 19 cows (yelding about 4200 kg milk per lactation), the latter being formerly included in search for the effect of AW season. Feeding was typical for each season: silages, hay, and concentrate in AW, and grass pasture, hay and concentrate in SS season. All the cows included were healthy and *mastitis*-free, and milk samples were obtained from each cow at the end of each season. The activity of both enzymes was found significantly lower in milk of cows at the end of AW than at the end of SS season. Since all the cows were found healthy and the difference could not be attributed to *mastitis*, it might be interpreted as an effect of seasonal feeding. It is suggested that the activity of both enzymes in milk of *mastitis*-free cows could be an indicator of efficiency of feeding system applied, and that the milk yielded during SS feeding is of higher cheese-making technological value for (e.g. Cheddar cheese production that requires dephosphorylation of protein) than that yielded during AW season. Further studies are needed to confirm our results.

KEY WORDS: acid phosphatase / cows / β-N-acetylhexosaminidase / enzymes / feeding

Milk is a complex composition reflecting the activities of distinct secretion and transport processes of the mammary gland reflecting the nutritional requirements of

mammalian neonates [Wiederschain and Newburg 2001, McManaman and Neville 2003, Jóźwik *et al.* 2004]. There are many bioactive peptides and proteins in milk, including enzymes which have not only nutritive, but also protective functions. Indigenous enzymes in milk may be used as indicators of its adequate pasteurization or of *mastitis.* Some of them were considered important for the stability of milk or related to its technological properties [Fox and Kelly 2006]. Generally, the lysosomal enzymes may play an important role in the adaptative physiological mechanisms to maintain the organism's homeostasis. Especially important of lysosomal enzymes are acid phosphatase (AcP) and β-N-acetylhexosaminidase (Hex) [Witek and Kołątaj 1998, Kołątaj *et al.* 2001].

AcP has been isolated from milk as a heat-stable compound (D-value of 10 min. at 75°C, pH 6.7) and has an optimum pH of 4.9°C what means that the enzyme is stable at temperature slightly higher than that required for killing milk pathogens and remains active in ripening cheese produced from pasteurized milk [Magboul and McSweeney 1999, Shakeel-Ur-Rehman *et al.* 2006]. AcP hydrolyses phosphoproteins (including caseins) causing their dephosphorylation. This was confirmed by Singh *et al.* [1997], who observed extensive dephosphorylation of some peptides originating from α-S1, α-S2 and β-caseins in Cheddar cheese. Some peptides produced during cheese ripening are rich in phosphate groups which, due to their protective effect, make the peptides resistant to further proteolysis [Fox *at al.* 1993]. Thus, the combined action, *i.e*. dephosphorylation of these peptides by phosphatase and further hydrolysis by proteolytic enzymes is required [Larsen and Parada 1988]. The AcP function of interest is particularly important in cheese ripening because of flavour development, which among others is a result of proteolysis [Fox and McSweeney 1996]. This biochemical event is also largely responsible for textural changes of cheese [Fox 1989]. The other role of AcP seems to be the participation in natural defence mechanisms against pathogens [Kaczmarczyk *et al.* 1999].

Hex is an acid hydrolase of cell lysosomes isolated from many animal and human tissues and constitutional fluids [Mellors 1968]. Most Hex activity in normal bovine milk comes from secretion by epithelial cells while less Hex is associated with mammary leukocytes [Fox *et al.* 1988]. Hex activity in milk increases during *mastitis*, reflecting epithelial cell damage and leukocyte infiltration, and its activity and somatic cell counts in milk are generally highly correlated [Kaartinen *et al.* 1990]. Therefore, also this enzyme was first used a**s** an indicator of *mastitis*. Hex contributes to the hydrolysis of bacterial walls and for this reason has a bactericidal effect against several bacterial pathogens [Hussain *et al.* 1992]. Hex is also involved in enzymatic formation of hyaluronate metabolites, in adhesive processes of cells and in connective tissue metabolism [Kankofer *et al.* 2000]. Harmon *et al*. [1975] showed that Hex is secreted from the blood capillaries directly into the milk as a defence reaction, similarly to lactoferrin.

In the available literature there is limited information concerning the effects of contrasting feeding systems as reflected by the season of the year on enzyme activities

in cows' milk. Thus, the aim of this study was to estimate the influence of feeding system on acid phosphatase and β-N-acetylhexosaminidase activities in the raw whole milk of cows.

Material and methods

Animals and feeding

Analysed were milk samples from Polish Red cows maintained on ecological farm, yielding about 4200 kg milk per lactation and fed according to the season of the year as follows: from October to May (autumn/winter – AW season) and from June to September (spring/summer – SS season). Over the AW season 23 cows were fed with conserved feeds, i.e. maize silage and grass silage (1:1 on dry matter basis), hay and concentrate mix (mainly cereals) with mineral-vitamin premix. Over the SS season the investigation was continued on 19 cows from the former AW group but kept on pasture and fed a supplement of hay and concentrate as in AW season. The diets were balanced according to INRA feeding standards. The difference in cows' number between AW and SS season was caused by the health problems occurring in the middle of SS season with four cows. Milk samples were taken once at the end of May, and at the end of September. Only cows with healthy udders according to actual Polish Standard for raw milk were considered.

The experiment was performed under an official permit from the 3rd Local Ethics Commission working at the Warsaw Agricultural University.

Analytical

The activities of acid phosphatase (AcP - EC 3.1.3.2), and β-N-acetylhexosaminidase (Hex – EC 3.2.1.52), were assayed according to the method of Barrett and Heath [1972], using substrates from SIGMA-ALDRICH Co. Ltd.

The enzyme activities were measured after incubation at 37°C and were expressed in nmol/mg of protein/hour. In milk samples the protein concentration was estimated by the method cited by Krawczyński and Osiński [1967] with bovine serum albumin as a standard. All biochemical assays were performed spectrophotometrically, using Lambda Bio20 (PERKIN-ELMER).

Fat, protein and lactose content was estimated in milk samples using Milko Scan 104A/B, and somatic cell counts (SCC) were determined with FOSSOMATIC device.

Statistical

The data were analysed using GLM procedure of SAS v. 8e [2001]. The following statistical model was used:

$$y_{ijk} = \mu + S_i + P_j + b_1(x_1 - DD)_{ijk} + b_2(x_2 - MY)_{ijk} + e_{ijk}$$

where:

y_{ijk} – activity of enzymes, content of protein, fat and lactose, and SCC;
μ – overall mean,;
S_i – fixed effect of season of feeding;
P_j – fixed effect of parity;
DD – mean number of days-in-milk from calving to sampling;
b_1 – linear regression coefficient on days-in-milk;
MY – mean milk yield;
b_2 – linear regression coefficient on milk yield;
e_{ijk} – random error.

The SCC values were transferred to logarithm scale.

Results and discussion

Almost all investigated effects appeared not significant upon the basic chemical composition of milk, SCC and activity of acid phosphatase (AcP) and β-N-acetylhexosaminidase (Hex) – Table 1. The only significant effects identified were those of feeding season on protein and lactose content ($P<0.05$) and both enzymes activity ($P<0.01$), as well as of parity on lactose content ($P<0.05$). No significant differences were found in SCC and fat content of milk between seasons.

Table 1. Results of variance analysis

Trait	Effect		Fixed regression	
	parity	season	milk yield	day-in-lactation
Log SCC	ns	ns	ns	ns
FAT (%)	ns	ns	ns	ns
Protein (%)	ns	*	ns	ns
Lactose (%)	*	*	ns	ns
AcP	ns	**	ns	ns
Hex	ns	**	ns	ns

*P≤0.05; **P≤0.01.

The concentration of both protein and lactose of milk was significantly higher in summer than in winter ($P\leq0.05$). Also the fat content was higher in summer season, but the interseason difference was not significant. As already mentioned, all cows were *mastitis*-free and showed no signs of udder inflammation, confirmed by low level of SCC both in AW and SS season (Tab. 2).

Table 2. Least squares means (LSM) and their standard errors (SE) for somatic cell count (SCC) and selected chemical components of milk across systems of feeding

System of feeding		Item			
		SCC[1]	protein (%)	fat (%)	lactose (%)
Autumn-winter (AS)	LSM	4.72	3.39[a]	4.40	4.86[a]
n=23	SE	0.27	0.01	0.37	0.03
Spring-summer (SS)	LSM	4.63	3.93[a]	4.84	4.96[a]
n=19	SE	0.32	0.02	0.47	0.04

[a]Within columns means bearing the same superscripts differ significantly at P≤0.05.
[1]Ln.

Table 3 presents the activity of AcP and Hex in milk samples of cows across feeding seasons. The differences between seasons were highly significant (P≤0.01). The activities of both estimated enzymes were by about 22 % higher at the end of SS than at the end of AW season. in pasture than in the winter when traditional feeding system was applied (Tab. 3).

Table 3. Least squares means (LSM) and their standard errors (SE) for enzyme activity in milk across systems of feeding

System of feeding		Enzyme	
		AcP	Hex
Autumn-winter (AS)	LSM	21.66[A] (100%)	18.61[A] (100%)
n=23	SE	0.88	0.60
Spring-summer (SS)	LSM	26.52[A] (122%)	22.63[A] (122%)
n=19	SE	1.04	0.72

[A]Within columns means bearing the same superscripts differ significantly at P≤0.01.

As no significant influence of investigated effects, except season of feeding, was identified upon the activity of both enzymes, the authors are of opinion that the main factor determining the activity of AcP and Hex was feeding. The literature available lacks information on this topic and the authors are not able to compare their results with those of the other research. One of the main factors affecting the activity of AcP is clinical or sub-clinical *mastitis*. Andrews and Alichanidis [1975] reported that the AcP activity in milk might increase even 4-10 fold during *mastitis*. In the present report the SCC was even less in summer than in winter. Higher (although not significant) fat content of milk in SS season could lead to the non-significantly low AcP activity. Indeed, according to Fox and Kelly [2006] near 80% of the AcP activity is found in the skimmed milk, but the specific activity of the enzyme is higher

in cream. The significantly higher lactose content of milk during summer feeding suggests that cows ration was sufficient in energy. It was also in accordance with significantly higher concentration of protein in milk at the end of AW season. It can contribute to higher concentration not only of total protein in milk but also of casein, which contains sulphhydryl (-SH) groups. This suggestion corroborates the earliest results of Bingham and Garver [1990] who showed that AcP activity depends to some extent on the number of sulphydryl groups. With no reference to factors which led to the differences in concentration of AcP between seasons, the higher activity of the enzyme in milk at the end of SS season is advantageous from the point of view of technological properties of milk for cheese-making. AcP causes dephosphorylation of some peptides and increased efficiency of operation of proteolytic enzymes [Larsen and Parada 1988]. This function is important and essential in cheese ripening for the development of flavour and textural changes [Fox 1989, Fox and McSweeney 1996]. The other role of AcP seems to be its participation in natural defence mechanisms against pathogens [Kaczmarczyk *et al.* 1999].

In this report significant increase in the activity of Hex in cows' milk was found in SS as compared to AW feeding season. The authors suppose that the increase of activity of Hex in milk of cows maintained on pasture (SS) was related to quality and quantity of feeds consumed by cows.

In the literature available no experimental results were found concerning the influence of feeding system of milking cows on activity of Hex in their milk. The hypothesis of the effect of feeding cows on the enzymes investigated seems extremely credible, as there were no statistically identified effects of parity, milk yield and stage of lactation on the activity of Hex. Also the lack of differences in SCC between seasons suggests that higher activity of Hex in milk during SS season was not related to them. The lack of subclinical or clinical *mastitis* was confirmed by low SCC in both seasons of investigations (about 25×10^4/ml in AW and 20×10^4/ml in SS season). A relationship between SCC and the activity of Hex in milk is quite well documented. Leitner *et al*. [2003] showed significantly higher (more than 200% difference) Hex activity of milk in sheep with infected than with uninfected mammary gland. Hex activity is reported to identify intramammary infections with *mastitis* pathogens and it correlates very closely with SCC [Pyörälä 2003]. Hex, among others biological active substances has a bactericidal effect against several bacterial pathogens [Hussain *et al*. 1992]. Harmon *et al*. [1975] showed that Hex is secreted from the blood capillaries directly into milk as a defence reaction, similarly to lactoferrin. Thus Hex has antimicrobial function in the mammary gland as well as in gut of neonate. Moreover, the activity of Hex is used as an indicator to assure that properties of pasteurised milk important of cheese ripening have not been lost [Ardö *et al.* 1999].

The spring/summer (SS) feeding system led to significant increase in the activity of acid phosphatase and β-N-acetylhexosaminidase of milk as compared to autumn/winter (AW) season and could probably positively affect the immunological system of mammary gland. Up to now the activities of these enzymes in milk are considered

as the indicators of health status of mammary gland. On the basis of results presented here the authors suggest that the activity of investigated enzymes in milk of *mastitis*-free cows could be an indicator of feeding system efficiency. It also means that the milk obtained during SS feeding system is more valuable for making some kinds of cheeses (e.g. Cheddar), which require dephosphorylation of protein. Further studies are needed to confirm these conclusions.

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Aktywność kwaśnej fosfatazy i β-N-acetylheksozoaminidazy w mleku krów w sezonie jesienno-zimowym i wiosenno-letnim

S t r e s z c z e n i e

Celem badań było ustalenie wpływu systemu żywienia i utrzymania krów na aktywność kwaśnej fosfatazy (AcP) i β-N-acetylheksozoaminidazy (Hex) w mleku. Enzymy te grają istotną rolę w procesach metabolicznych zwierząt, a ich aktywność wpływa na parametry technologiczne mleka. Uważa się je za główne wskaźniki zaburzeń homeostazy oraz czułe wskaźniki stanu zapalnego wymienia.

Badania przeprowadzono na krowach rasy polskiej czerwonej, o średniej wydajności 4200 kg mleka za laktację, utrzymywanych na fermie ekologicznej. Wyróżniono dwa sezony żywienia – żywienie

typowe dla okresu jesienno-zimowego (sezon AW) i dla okresu wionenno-letniego (sezon SS). W sezonie AW zadawano 23 krowom kiszonkę z kukurydzy, kiszonkę z traw, siano łąkowe oraz paszę treściwą W następnym sezonie – SS – 19 krów z tej samej grupy pasiono na pastwisku, zadając dodatkowo po około 1 kg siana oraz zależnie od wydajności mleka – pasze treściwe. Wszystkie oznaczenia przeprowadzono na krowach ze zdrowym wymieniem.

Aktywność obu badanych enzymów w mleku okazała się istotnie wyższa w sezonie wiosenno-letnim (SS) niż w jesienno-zimowym (AW). Stwierdzona istotnie wyższa aktywność AcP i Hex w sezonie SS niż w AW nie mogła wynikać ze stanu zdrowia wymienia (wszystkie badane krowy były wolne od *mastitis*), ale jedynie (a przynajmniej głównie) z sezonu żywienia. W sezonie SS aktywność obu antybakteryjnych enzymów, a tym samym prawdopodobnie wydajność systemu obronnego zwierząt, była istotnie większa niż w sezonie AW. Na podstawie uzyskanych wyników autorzy sugerują, że aktywność badanych enzymów w mleku krów ze zdrowym wymieniem może być wskaźnikiem jakości systemu żywienia. Znaczy to również, że mleko pozyskiwane w sezonie pastwiskowym charakteryzuje się większą przydatnością do produkcji niektórych gatunków sera (np. Cheddar), których cechy jakościowe zależą od stopnia defosforylacji białek. Dla potwierdzenia przedstawionych sugestii niezbędne są dalsze badania.