

# Activity of N-acetyl- $\beta$ -D-hexosaminidase (HEX) and its isoenzymes A and B in human milk during the first 3 months of breastfeeding

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## ABSTRACT

**Purpose:** Milk contains free and bound oligo- and heteropolisaccharides, which protect newborns against pathogens and have nutritional value. N-acetyl- $\beta$ -D-hexosaminidase (HEX), the most active lysosomal exoglycosidase, modify and degrade oligo- and heteropolysaccharides. The objective of our study was to determine HEX activity and isoenzymes A and B in the progression of lactation.

**Material and methods:** Human milk samples were collected from 51 women on the 3<sup>rd</sup>, 21<sup>st</sup> and 100<sup>th</sup> day postpartum. Enzymatic activity was determined the Zwierz et al method modified by Marciniak et al. Protein and lactose concentrations were determined by a MilkoScan 4000 apparatus.

**Results:** The total HEX activity decreased by the 21<sup>st</sup> day in comparison to the 3<sup>rd</sup> day, and increased by the 100<sup>th</sup> day as compared to the 21<sup>st</sup> day. HEX A activity decreased by the 21<sup>st</sup> and the 100<sup>th</sup> day as compared to the 3<sup>rd</sup> day. HEX B activity decreased by 21<sup>th</sup> day and has the tendency to decrease by the 100<sup>th</sup> day as compared to the 3<sup>rd</sup> day. Protein concentration decreased and the lactose concentration increased in milk taken on the 21<sup>st</sup> day in comparison to concentration of protein and lactose on the 3<sup>rd</sup> day. HEX and its isoenzymes' activity significantly correlate with the progression of lactation. At the beginning of lactation, HEX A activity, which releases hexosamines from acidic oligosaccharides, dominates; later, HEX B releases hexosamines from neutral oligosaccharides.

**Conclusions:** To better understand the degradation of human milk oligosaccharides, it would be useful to investigate and document their detailed structures and evaluate the activity of other exoglycosidases' activity in human breast milk over the course of lactation.

**Key words:** human milk, breastfeeding, N-acetyl- $\beta$ -D-hexosaminidase, glycoconjugates

## INTRODUCTION

Human milk contains large amounts of both free (i.e. released from glycoconjugates) and bound oligosaccharides of glycoconjugates (glycoproteins, glycolipids and proteoglycans) [1-3]. Free and conjugated oligo- and heteropolysaccharides are not only a source of carbohydrates, but also biologically active milk components that inhibit pathogens by competitive binding to the host cell-surface receptor [1,4,5]. The biological

action of free and bound milk oligosaccharides depends on their composition and structure [3,6-8], which reflects the genetic information of milk producing cells as well as the action of the mammary gland and milk exoglycosidases [5].

N-acetyl- $\beta$ -D-hexosaminidase (HEX) catalyse the release of N-acetylglucosamine and N-acetylgalactosamine residues from the non-reducing end of free and bound oligosaccharide chains and is the most active of acid lysosomal exoglycosidases [9]. Isoenzymes of HEX there are dimers composed of two

polypeptide chains  $\alpha$  or  $\beta$ : S ( $\alpha\alpha$ ), A ( $\alpha\beta$ ), B ( $\beta\beta$ ), respectively [10].

The aim of our investigation was determination of the activity of N-acetyl- $\beta$ -D-hexosaminidase (HEX) and its isoenzymes A and B in human milk in the progression of lactation and to obtain information concerning possible changes in the structure and function of human milk oligosaccharides.

## MATERIAL AND METHODS

Samples of human milk were collected from 51 healthy women, mean age 27.55 years (16 primigravida's and 33 multiparous females), who born on time, in good general condition 24 boys (47%) and 27 girls (53%).

The following were the criteria of test exclusion:

- Jaundice as a result of asphyxia, sepsa, intrauterine infection, diabetes, serological incompatibility, subperiosteal haematoma and similar reasons;
- A body mass decrease of over 5 %, during the stay in the neonatal department;
- Additional nourishment of infant with a cow milk mixture;
- Oral or parenteral hydration; and,
- Mothers who did not deliver samples on the 3<sup>rd</sup>, 21<sup>st</sup> and 100<sup>th</sup> days after childbirth and dropped out of the study.

From each of 51 woman, 2 ml samples of milk were taken manually between 6-8 o'clock in the morning, on the 3<sup>rd</sup> (colostrum), and 21<sup>st</sup> (early milk) days postpartum as well as on the 100<sup>th</sup> day (mature milk), before the baby was fed. Milk was immersed in polyethylene tubes containing 1 ml ethylene glycol. Samples were centrifuged at 10,000 g for 10 minutes at 4°C. Supernatants were immediately frozen and stored at -80 °C until analysis. Directly before the analysis, the frozen samples were thawed at room temperature.

N-acetyl- $\beta$ -D-hexosaminidase (HEX) and its isoenzyme A and B activity (mmol/ml/min) were determined by the Zwierz *et al.* method [11] with Marciniak *et al.* modification [12].

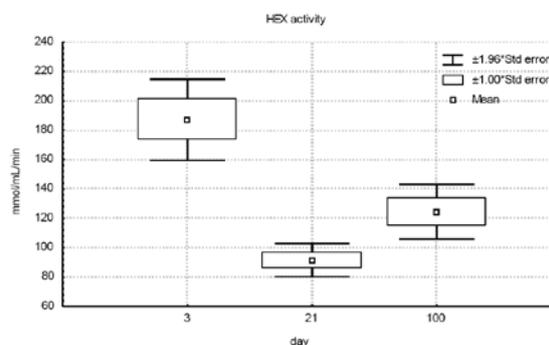
Milk samples were diluted 10 times with deionized water. All tests were conducted in duplicates.

The study design was approved by the Ethical Committee of the Medical University of Bialystok, Poland. Consent of mothers and permission for the study was obtained in accordance with the guidelines of the Ethics Committee of Medical University of Bialystok, Poland.

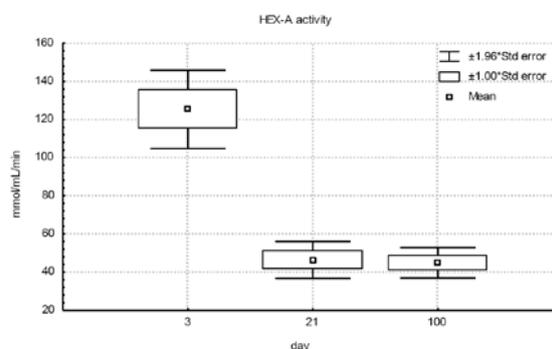
### Statistical analysis

The results were analysed by a Statistica 6.0 StatSoft (StatSoft Polska, Krakow, Poland) according to the Kruskal-Wallis test. Statistical significance of differences was regarded at  $p < 0.05$ .

**Figure 1.** N-acetyl- $\beta$ -D-hexosaminidase (HEX) activity 3<sup>rd</sup>, 21<sup>st</sup> and 100<sup>th</sup> day after postpartum.



**Figure 2.** Isoenzyme A activity 3<sup>rd</sup>, 21<sup>st</sup> and 100<sup>th</sup> day after postpartum.



## RESULTS

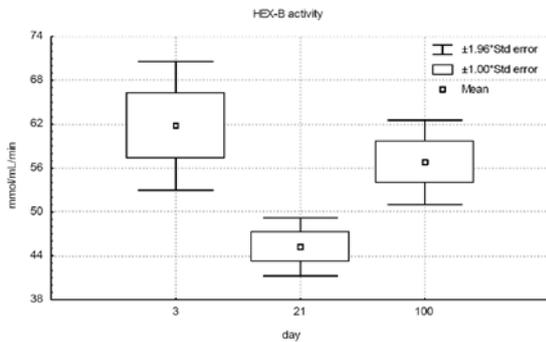
Our results show that the total HEX activity significantly decreased by the 21<sup>st</sup> day of lactation, as compared to milk taken on the 3<sup>rd</sup> day of lactation ( $p = 0.000001$ ), and significantly increased by the 100<sup>th</sup> day after postpartum, in comparison to activity on the 21<sup>st</sup> day of lactation ( $p = 0.015246$ ). Total HEX activity significantly decreased by the 100<sup>th</sup> day of lactation, as compared to the milk taken on the 3<sup>rd</sup> day of lactation ( $p = 0.002358$ ) (Fig. 1).

The activity of HEX A significantly decreased by the 21<sup>st</sup> ( $p = 0.000001$ ) and 100<sup>th</sup> ( $p = 0.000060$ ) day of lactation as compared to the 3<sup>rd</sup> day of lactation (Fig. 2). There were no significant changes in HEX A activity between milk taken at 21<sup>st</sup> and 100<sup>th</sup> day of lactation ( $p = 0.305205$ ) (Fig. 2).

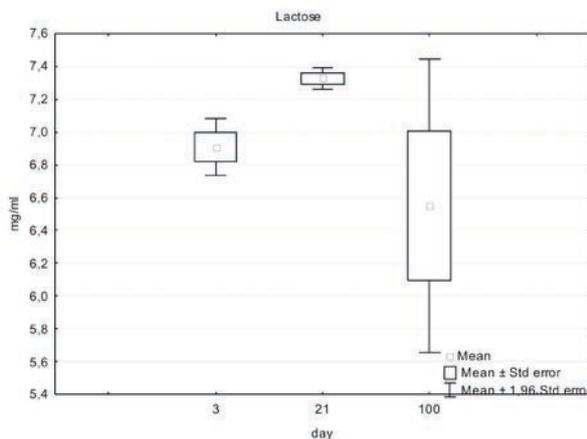
The activity of HEX B significantly decreased on the 21<sup>st</sup> day of lactation as compared to activity in milk obtained on the 3<sup>rd</sup> day ( $p = 0.000511$ ), and we observed the tendency to decrease HEX B activity at the 100<sup>th</sup> day of lactation in comparison to the 3<sup>rd</sup> day ( $p = 0.394463$ ). The activity of HEX B significantly increased by the 100<sup>th</sup> day of lactation as compared to the 21<sup>st</sup> day ( $p = 0.014978$ ) (Fig. 3).

The concentration of protein in milk significantly decreased by the 21<sup>st</sup> and 100<sup>th</sup> days of lactation in comparison to the 3<sup>rd</sup> day of lactation with ( $p = 0.0006$ ) and ( $p = 0.0268$ ), respectively (Fig. 4). The concentration of milk lactose significantly

**Figure 3.** Isoenzyme B activity 3<sup>rd</sup>, 21<sup>st</sup> and 100<sup>th</sup> day after postpartum.



**Figure 5.** Lactose concentration in human milk on 3<sup>rd</sup>, 21<sup>st</sup> and 100<sup>th</sup> day after postpartum.

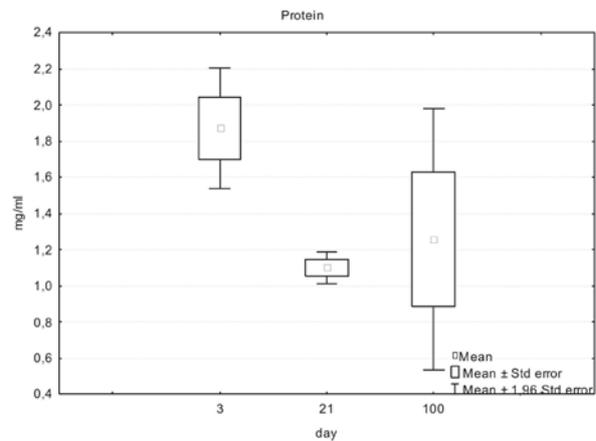


( $p=0.0461$ ) increased by the 21<sup>st</sup> day of lactation, as compared to activity in milk obtained on the 3<sup>rd</sup> day of lactation, and remained at the same level through the 100<sup>th</sup> day of lactation ( $p=0.1758$ ) (Fig.5).

## DISCUSSION

The nutritional value and the interactions with pathogens [1,4,5] of free and bound milk oligosaccharides depend on their composition and structure [3,6-8]. The release of free oligosaccharides and the modification of their oligosaccharide chains result from the activity of endo- and exoglycosidases ( $\alpha$ -L-fucosidase,  $\alpha$ -D-galactosidase,  $\beta$ -D-galactosidase,  $\beta$ -D-glucosidase, N-acetyl- $\beta$ -D-hexosaminidase,  $\beta$ -D-glucuronidase and neuraminidase [5,13,14]. N-acetyl- $\beta$ -D-hexosaminidase and  $\alpha$ -L-fucosidase (which displayed the highest activity), were detected in the samples of human milk. Wiederschain and Newburg (2001) presented proof that  $\alpha$ -L-fucosidase, N-acetyl- $\beta$ -D-hexosaminidase, and neuraminidases in human milk participate in hydrolytic modifications of free and bound oligosaccharides and the release of free sugars.

**Figure 4.** Protein concentration in human milk on 3<sup>rd</sup>, 21<sup>st</sup> and 100<sup>th</sup> day after postpartum.



Glycoconjugates are products of biosynthetic and degradation processes that take place in the mammary gland during lactation. The biological activity of glycoconjugates depends upon the precise structure of the glycan [4,5,7]. In human milk, there are both substrates and products of HEX action [3]. We found a significant decrease of total HEX activity on the 21<sup>st</sup> day of lactation in comparison to the 3<sup>rd</sup> day, and a significant increase in 100<sup>th</sup> day of lactation in comparison to 21<sup>st</sup> day of lactation (Fig. 1). The biological significance of this decrease in HEX activity in milk on 21<sup>st</sup> day of lactation is difficult to explain. It may be speculated that the decrease in milk HEX activity on 21<sup>st</sup> day of lactation may slow down the destruction of biological activity of oligosaccharide chains of glycoconjugates important in some immunological processes, which depend on terminal N-acetylgalactosamine (as A blood group substance) or N-acetylglucosamine moiety. It was reported that some human milk oligosaccharides have a protective activity against pathogens, even in lower concentrations than found in human milk [5,7,15]; therefore, one can speculate that milk HEX activity may modify the structure and biological activity of milk oligosaccharides containing hexosamines, adjusting their structure to the actual needs of the baby.

The increase of HEX activity (which release free hexosamines) in milk from 21<sup>th</sup> to the 100<sup>th</sup> days of lactation in comparison to HEX activity on the 21<sup>th</sup> day of lactation may have nutritional value, because the concentration of hexosamines is only one order of magnitude below human milk glucose [5]. A decrease in protein (Fig.4) and an increase in lactose (Fig.5) concentration of human milk in the course of lactation may suggest that, as the development of the baby proceeds, energy supply is more important than the supply of proteins.

Since the total HEX consists of HEX A and HEX B activity [10], we determined HEX A and HEX B activity in the course of lactation. We found a significant decrease in HEX A milk activity on the 21<sup>st</sup> day and a tendency to increase on the 100<sup>th</sup> day of lactation in comparison to the 3<sup>rd</sup> day of

lactation (Fig.2), which is in agreement with Oberkotter *et al.* [8] report. We demonstrated a significant decrease HEX B activity on the 21<sup>st</sup> day and a tendency to decrease HEX B activity by the 100<sup>th</sup> day of lactation in comparison to the 3<sup>rd</sup> day of lactation (Fig.3). Changes in HEX isoenzymes activity may have an influence on the composition of oligosaccharide chains of human milk as HEX A release hexosamines from acidic oligosaccharides [16], and HEX B acts on neutral substrates [17,18]. It is hypothesized that HEX may be part of the normal complement of catabolic enzymes found in phagocytic leucocytes. The level of leucocytes in milk, which are a rich source of lysosomal enzymes, varies according to many factors, including the stage of lactation [4,5,7]. This may relate to our data concerning the preponderance of different isotypes of enzymes early and late in lactation. Our results suggest that, at an early lactation period in human milk, there are conditions to release hexosamines from acidic oligosaccharides (represented by HEX A activity). Then, as lactation proceeds, there is a growing possibility to release hexosamines from neutral oligosaccharides (represented by HEX B activity). The physiological relevance of this fact remains to be established.

## CONCLUSIONS

In conclusion, it was found that the activity of total HEX as well as HEX A and HEX B correlate with the progression of lactation. At the beginning of lactation, HEX A, releasing hexosamines from acidic oligosaccharides, dominates, and later HEX B, releasing hexosamines from neutral oligosaccharides, dominates. However, information about glycosidase activity in human milk is very limited; therefore, to have clear insight into the degradation of human milk oligosaccharides, it would be valuable to investigate and identify their detailed structures, and also to evaluate exoglycosidases activity in human breast milk acting on particular oligosaccharides over the course of lactation.

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