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The effect of dietary protein level on selected degradative enzymes activity of hepatocyte cellular subfractions in experimental mice

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Studied was the activity of selected degradative enzymes of lysosomal, microsomal and cytoplasmic fraction of hepatocytes as affected by different protein levels in the diet of experimental mice. The control animals were fed with standard 16% protein level in the diet, while experimental animals with low (10%) or high (40%) protein diet. The animals were fed *ad libitum*. Changes in fractions depended on the kind of enzyme, cell fraction and protein level in examined diets. The high protein diet increased activity of six enzymes in the lysosomal fraction and decreased activity of β-Gal only, while the low protein diet caused an increase of activity of this fraction of seven enzymes. The 40% protein diet decreased the activity of nine enzymes in the microsomal fraction, the AcP did not change only. The 10% protein diet in this fraction decreased the activity of seven enzymes and increased activity of two enzymes. In the cytoplasmic fraction, the high protein diet decreased the activity of eight enzymes. Higher activity was observed only for AcP. The low protein diet caused a decrease in seven enzymes activity, and an increase of β-GlcUr activity only. The change of normal standard protein diet in animal feeding on its high or low-protein level occurred to be a significant factor disordering the biochemical homeostasis of the cell.

KEY WORDS: hepatocytes / lysosomal enzymes / mice / protein diet

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The most effective enzymatic degradation system in the cell is localized in its lysosomes that play an important role in adaptation processes of cell digestive system [Jóźwik *et al*. 2013, Kołątaj *et al*. 2001, Marschner *et al.* 2011, Schultz *et al.* 2011, Witek *et al*. 2007].

The hydrolysis of organic different macromolecules, especially proteins may also be found in lysosomal, microsomal and cytoplasmic fractions [Grune *et al*. 1997, Staszczak and Zdunek 1999, Witek *et al.* 2011].

The changes of the protein level in an animal and human diets can affect the differences of enzyme activities in the tissues. They can be analysed from the point of view of environmental starvation stress leading to changes in oxidative status of animals where the quantity of generated free radicals in much higher than antioxidants [Jóźwik *et al*. 2010] and – on the other hand – high, abnormal quantities of protein in the food met in human nutrition.

The aim of this study was to identify some changes related to the activity of some hydrolytic enzymes in lysosomal, microsomal and cytoplasmic fractions of hepatocytes under the influence of various protein levels in the diets of mice.

Material and methods

The study was performed on Swiss male mice, aged 8-9 weeks, with a mean body weight 25.0±1.3 g, randomly selected from the mice farm of the Institute of Genetics and Animal Breeding, Polish Academy of Sciences in Jastrzębiec, Poland. Animals were kept in standard cages in housing room with a temperature of 20-22°C, with 12-hour light cycle (LD 12:12), and *ad libitum* access to granulated feed and water. After weaning at the age of 6 weeks, the animals were allocated to the three feeding groups with protein levels 16%, 40% and 10%. All diets were prepared especially for this experiment, according to the composition and nutritive value given in Table 1, prepared by Animal Feed Company in Łomna-Las Farm near Warsaw. All animals had free access to feed and water. The diets differed in protein levels but energetic values were similar (Tab. 1).

All animals were maintained in good physical conditions and remained under careful veterinary control. They were divided into three nutritional groups, consisting of 30 animals each:

1. Animals receiving a standard diet, with 16% protein content (control group);
2. Animals kept on high-protein diet containing 40% protein;
3. Animals kept on low-protein diet containing 10% protein.

After 15 days, animals fed with the above diets were decapitated after short narcosis by vetbutal. The liver was immediately perfused in 0.9% solution of NaCl at 5°C, and placed in 0.25 M saccharose solution with 2 mM EDTA addition, in proportions of 1 gram of tissue per 6 ml solution. Liver slices were homogenized in the Potter-Elvehjem homogenizer with a Teflon piston at 200 rpm. Liver homogenates were centrifuged with Janetzky K26D (8 min. at 5500 rpm), Sorvall RC-5C (20 min.

Table 1. Composition, nutritive value of protein, and gross energy content of the diets

Composition	Percentage of protein in diet		
	Standard diet (16%)	40%	10%
Premix LSM	1.01	0.97	1.02
Salt	0.29	0.28	0.29
Chalk	1.64	1.57	1.65
Phosphates	0.97	0.87	0.97
Milk powder	5.80	11.53	4.83
Soybean meal	10.14	48.38	1.94
Fish meal	-	15.66	-
Yeast	-	5.07	-
Cereal germs	15.44	15.67	
Corn meal	64.71	-	89.30
Gross energy (MJ/kg)	~14.04	~15.38	~13.47

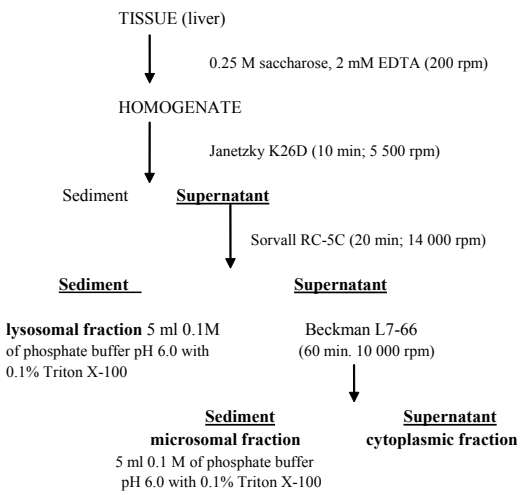

Fig. 1. Differential centrifuging according to Marzella and Glaumann [1980].

at 14 000 rpm), and Beckman L7-66 centrifuge (60 min. 10 000 rpm) according to Marzella and Glaumann [1980] – Figure 1.

The preparates of lysosomal and microsomal sediment after suspension in 0.1 M phosphate buffer, pH 7.0 with the addition of 0.1% Triton X-100 were twice frozen and refrozen and centrifuged (10 min.700g) in the Janetzky K26D. The cytoplasmic fraction, after double freezing and refreezing was directly ready for the assays.

For each fraction received lysosomal, microsomal and cytoplasmic (according to the data from Tab. 2) enzyme activity was determined with the use of their respective substrates.

Table 2. Lysosomal enzymes examined

Enzyme	Number of enzyme	Method
Glycosidase hydrolases		
β-glucuronidase (β-GlcUr)	EC 3.2.1.31	Barrett and Heath [1977]
β-galactosidase (β-Gal)	EC 3.2.1.23	Barrett and Heath [1977]
β-glucosidase (β-Glu)	EC 3.2.1.21	Barrett and Heath [1977]
N-acetyl-hexosaminidase (Hex)	EC 3.2.1.52	Barrett and Heath [1977]
Carboxylic ester hydrolases		
Lysosomal esterase (EL)	EC 3.1.1.2	Barrett and Heath [1977]
Lysosomal lipase (LL)	EC 3.1.1.13	Barrett and Heath [1977]
Phosphate monoester hydrolase		
Acid phosphatase (AcP)	EC 3.1.3.2	Barrett and Heath [1977]
Peptydyl-peptide hydrolases		
Cathepsin D (Cath. D)	EC 3.4.23.5	Langner *et al*. [1973]
Cathepsin L (Cath. L)	EC 3.4.22.15	Langner *et al*. [1973]
α-aminoacyl-peptide hydrolases		
Leucine aminopeptidases (LeuAP)	EC 3.4.11.1	Mc Donald and Barrett [1986]
Alanine aminopeptidases (AlaAP)	EC 3.4.11.2	Mc Donald and Barrett [1986]

The activities of β-GlcUr, β-Gal, β-Glu, Hex, EL, LL and AcP were determined spectrophotometrically as 4-nitrophenyl derivatives at 420 nm according to Barrett's and Heath's [1977] micro-method. The activity of Cath. D and L were determined with 6% azocasein derivatives at 366 nm according to Langner *et al*. [1973]. The activity of LeuAP and AlaAP was determined as Fast Blue BB salt [4-benzoylamino-2.5-diethoxybenzene-diazonium chloride] derivatives at 540 nm according to the method of Mc Donald and Barrett [1986].

The protein was determined according to a modified method of Kirschke and Wiederanders [1984]. All substrates were produced by SERVA FEINBIOCHEMICA GmbH& Co (Heidelberg, Germany). The activity of enzymes studied has been expressed in nmol/mg protein/hour.

Multifactor analysis of variance of the results obtained was conducted with the use of SAS/STAT Software [1999-2001, User's Guide, SAS Institute Inc., Cary NC, USA] and Origin [version 5.0, Microcal Software Inc., Northampton, USA].

This experiment was approved by the Commission for Ethics in Experiments on Animals of the Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Jastrzębiec.

Results and discussion

It is known that with an excess availability of protein in the diet, the release of amino acids causes an increase of the ammonia production, resulting in an increased excretion of urea and increased probability of aminoaciduria occurrence. Raised levels of amino acids in the blood can also be the reason of specific metabolic disorders,

intensifying for example the oxidation stress, especially with regard to tyrosine and sulphur amino acids – cysteine and methionine [Caylak *et al.* 2008, Dröge 2005, Tyagi *et al.* 2005]. Conversely, the literature concerning caloric restrictions suggests that a low caloric diet can extend life [Edwards *et al.* 2010, Smith *et al.* 2010, Turner and Iwaniec 2010], although when a cell is deficient in energy, its functions can be impaired.

The results of our investigations (Tab. 3-6) revealed that the activity of the studied enzymes changed multidirectionally. It was dependent on the enzyme and diet with different protein content, as well as upon the cell fraction. Significant changes in the content of protein in animal and human diets could also be interpreted based upon the stress categories, especially in the case of malnutrition or protein starvation and also in the case of its excess on the other hand [Hardie 2011, Nakahara *et al.* 2010, Shang *et al*. 2011]. The enzymes of cell subfractions – whose activity changed in these

Table 3. The activity [nmol/mg protein/h] of lysosomal aminopeptidases and proteases in lysosomal, microsomal and cytoplasmic fraction of the mouse hapatocytes after application of different protein diets

Enzyme	Fraction	16% protein	40% protein	10% protein
AlaAP	L	95.6±9.7	137.5±11***	238.7±20***
	M	208.2±21	84.6±7.3***	221.2±18^{ns}
	C	134.1±11	80.7±7.8***	74.4±6.0***
LeuAP	L	146.8±15	167.2±14^{ns}	261.8±4.0***
	M	232.5±23	69.5±5.6***	177.7±0.2***
	C	75.1±6,1	55.7±4.5**	50.5±0.2***
Cath. D and L	L	22.7±2.4	33.6±2,6***	49.6±4.0***
	M	3.0±0.1	1.8±0.3***	4.7±0.2***
	C	0.89±0.2	0.38±0.1***	1.0±0.2^{ns}

*P≤0.05; **P≤0.01; ***P≤0.001; ns – not significant.

Table 4. The activity (nmol/mg protein/h) of AcP and lysosomal lipases in lysosomal, microsomal and cytoplasmic fraction of the mouse hapatocytes after application of different protein diets

Enzyme	Fraction	16% protein	40% protein	10% protein
AcP	L	1979.4±198	3148.1±262***	2834.5±235***
	M	795.7±79	719.9±60^{ns}	965.8±81*
	C	1520.0±127	2679.4±223***	1330.4±111^{ns}
EL	L	1434.1±143	1630.0±136^{ns}	1434.3±120^{ns}
	M	864.9±86	187.0±15***	451.7±37***
	C	320.5±27	155.5±13***	133.8±11***
LL	L	574.8±58	1511.5±126***	622.8±52^{ns}
	M	592.6±59	402.6±33***	434.0±36**
	C	134.2±11	129.3±11^{ns}	96.6±7.9**

*P≤0.05; **P≤0.01; ***P≤0.001; ns – not significant.

Table 5. The activity [nmol/mg protein/h] of lysosomal glycosidases in lysosomal, microsomal and cytoplasmic fraction of the mouse hapatocytes after application of different protein diets

Enzyme	Fraction	16% protein	40% protein	10% protein
β-GlcUr	L	303.1 ± 30	418.0 ± 35***	564.4 ± 47***
	M	94.0 ± 9.2	38.6 ± 3.1***	61.6 ± 5.0***
	C	62.4 ± 5.0	25.8 ± 2.0***	127.0 ± 10***
β-Gal	L	347.8 ± 35	277.4 ± 23*	567.9 ± 48***
	M	59.5 ± 5.8	25.3 ± 2.0***	26.6 ± 2.1***
	C	325.5 ± 27	78.1 ± 6.3***	68.5 ± 5.6***
β-Glu	L	117.1 ± 12	153.7 ± 13***	341.8 ± 28***
	M	23.9 ± 2.2	5.5 ± 0.3***	8.6 ± 0.6***
	C	134.0 ± 11	54.8 ± 4.4***	79.3 ± 6.4***
Hex	L	842.1 ± 84	769.6 ± 64^{ns}	724.0 ± 60^{ns}
	M	287.1 ± 29	180.6 ± 14***	171.5 ± 14***
	C	225.0 ± 19	68.1 ± 5.5***	102.0 ± 8.3***

*P≤0.05; **P≤0.01; ***P≤0.001; ns – not significant.

Table 6. The effect of protein diets on the activity of examined enzymes (globally) in lysosomal, microsomal and cytoplasmic fraction of the mouse hepatocytes

Fraction	40% protein in diet			10% protein in diet		
	increase	without changes	decrease	increase	without changes	decrease
Lysosomal	6	3	1	7	3	-
Microsomal	-	1	9	2	1	7
Cytoplasmic	1	1	8	1	2	7
Total	7	5	18	10	6	14

conditions – showed expressive adaptation abilities with regard to the control values of diet containing 16% protein. To summarize, different content of protein applied in the experimental diets caused specific changes, namely increased or decreased activity of the studied enzymes.

In tables 3-5, it can be observed that the high protein diet (40%), increased activity of six enzymes studied in the lysosomal fractions – AlaAP, Cath. D and L, AcP, LL, β-GlcUr, β-Glu; decreased activity only one, namely β-Gal and did not cause any change in the activity of the three enzymes LeuAP, EL and Hex. The low protein diet (10%) caused – in comparison to the control diet – an increase of activity in the lysosomal fraction of seven enzymes – AlaAP, LeuAP, Cath. D and L, AcP, β-GlcUr, β-Gal and β-Glu. However, it did not cause any change in the activities of EL, LL and Hex.

The high protein diet did not increase the enzyme activity of the microsomal fraction, it decreased the activity of nine enzymes – AlaAP, LeuAP, Cath. D and L, EL, LL, β-GlcUr, β-Gal, β-Glu and Hex, not causing any changes in one of them – AcP. The low protein diet in this fraction decreased the activity of seven enzymes – LeuAP, EL, LL, β-GlcUr, β-Gal, β-Glu and Hex. It did not change the activity of

AlaAP only and increased activity of Cath. D and L and AcP only.

In the cytoplasmic fraction, the high protein diet lowered the activity of AlaAP, LeuAP, Cath. D and L, EL, β-GlcUr, β-Gal, β-Glu and Hex equaling eight enzymes. Higher activity was observed only for AcP, while for LL no changes were observed. The low protein diet [10%] caused a decrease in the activity of seven enzymes in this fraction - AlaAP, LeuAP, EL, LL, β-Gal, β-Glu and Hex, an increase of β-GlcUr activity and did not change Cath. D and L as well as AcP activity.

From a total of thirty possibilities predicted, a high protein diet increased the activity of the enzymes studied in all three fractions seven times, decreased in eighteen cases and caused no statistically significant changes in five times.

The low protein diet significantly increased the activity of the enzymes studied in ten cases, decreased it in fourteen and did not show any differences in six of them. We can, presume that both the high and low protein diets essentially decreased the activity of the studied degradation enzymes, although more distinctly in the case of the high protein diet. The combined data are presented in Table 6.

Certain trends of these changes were observed in relation to a displacement between fractions. With regard to the lysosomal fraction, both high and low protein diets significantly increased the activity of the enzymes tested (six and seven cases, respectively, from a possible ten), a decrease of activity was only once observed. In microsomal fraction a decrease of this activity of nine and seven times was observed at high and low protein diet, respectively, in the case of cytoplasmic fraction eight and seven times, respectively. The increase was observed here once in one time, respectively.

It can be stated that in the case of thirty possible comparisons tested, fourteen times a decrease was observed under low diet conditions, while in eighteen did not under high protein diet.

In comparison to a standard amount of protein in the diet, an increased or decreased its level revealed the displacements of enzyme activity in a selective manner, usually with a growing trend for the lysosomal fraction and a diminishing tendency for the microsomal and cytoplasmic fractions. From a total of 30 possible comparisons, it was only in 5 and 6 cases, respectively, that no activity changes were observed. However it was an observation with regard to different enzymes.

In the case of both increased and decreased dietary protein levels, such enzymes as AlaAP, Cath. D and L, AcP, β-GlcUr and β-Glu consistently increased in lysosomal fraction their activity, EL and Hex did not change and only β-Gal significantly decreased its activity, however the last observation was made in relation to animals who received the high protein diet. In the microsomal fraction, for the low protein diet, there were only two enzymes which increased their activity – Cath. D and L and AcP, while enzymes LeuAP, EL, LL, β-GlcUr, β-Gal, β-Glu and Hex decreased.

In the cytoplasmic fraction – only two enzymes increased their AcP activity – for animals who were fed with the high protein diet and β-GlcUr for animals receiving the low protein alternative. Three of the enzymes did not show any changes in their

activity – Cath. D and L and AcP for low protein diet groups and LL for the high protein.

Summarizing it can be concluded that the high protein level of diet did not stimulate sufficiently the activity of degradative enzyme system but rather decreases it, particularly in microsomal and cytoplasmic fractions of cell. The low protein level in diet decreases markedly the enzyme activity in microsomal and cytoplasmic fractions but increases it in lysosomal fractions. Such charges aim at assuring the optimum protein degradation rate in the cell according to its actual needs.

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