

Faculty of Medicine, Novi Sad  
Department of Biochemistry

Originalni naučni rad  
*Original study*  
UDK 618.19-006.03/04-074:577.15  
DOI: 10.2298/MPNS1206200B

## COMPARISON OF GLYCOLYTIC ENZYME AND ISOENZYME ACTIVITY IN BREAST CANCERS AND DYSPLASIA

*KOMPARACIJA AKTIVNOSTI GLIKOLITIČKIH ENZIMA I IZOENZIMA KARCINOMA I DISPLAZIJA DOJKE*

**Katica BAJIN KATIĆ**

**Summary** – The study was aimed at assessing the total enzyme activity and the profile of breast cancer and dysplasia on the human material. In addition, the validity of data was evaluated from the aspect of improving diagnostics. Lactate dehydrogenase activity, as well as the profile of its isoenzymes, pyruvate kinase and hexokinase, were measured. The study included 60 samples of breast cancer, out of which 20 were benign breast tumours and 40 were 1<sup>st</sup> and 2<sup>nd</sup> degree dysplasia of the breast. The samples were collected from the patients operated at the Institute for Oncology of Faculty of Medicine in Sremska Kamenica. Lactate dehydrogenase isoenzymes were separated by the vertical polyacrylamide gel disc electrophoresis according to the slightly modified Brewer and Ashworth's method. The activity of all the tested enzymes was measured under the conditions of linear kinetics in the function of time and enzyme concentration. Lactate dehydrogenase-5 was found in 88% of the analyzed breast cancer samples, whereas it was not detected in breast dysplasia. Pyruvate kinase (4.-isoenzyme) was about 50 times higher and the activity of hexokinase was 3 times higher in breast cancer than in breast dysplasia. Lactate dehydrogenase-5 and pyruvate kinase (4.-isoenzyme) are particularly important and reliable markers of malignity. The results obtained for quantitative and qualitative changes in the enzyme activity can be used to improve diagnostics and early diagnostics of malignant breast neoplasm.

**Key words:** Breast Neoplasms; Fibrocystic Breast Disease; Biological Markers; L-Lactate Dehydrogenase; Pyruvate Kinase; Hexokinase; Diagnosis; Early Detection of Cancer

### Introduction

Several important hypotheses have been set when attempting to explain biochemistry of neoplasm and to find molecules that would be specific for tissues growing uncontrollably. All the observations, which are basically correct, point to some specific features of malignant neoplasm; however, none of the hypotheses resulting from the above observations [1-4] could explain the true nature of the very complex neoplastic process.

Attempts at proving differences between a normal and malignantly transformed cell have failed so far, and only few researchers believe that tumour tissues are characterized by certain biochemical features that could not be found in normal cells [2].

Nowadays, the association between malignant neoplasm and regulation disorders, i.e. gene expression, is being talked about with more confidence. The significant changes occurring during malignant alteration are closely related with the new programme of protein synthesis in tumour cells [5-12]. The reprogramming of gene expression results in the changes in the biomechanics of malignant cell as well. Gene expression in tumour cells is unpredictable and extremely heterogeneous.

Since enzymes are the products of genes, the quantitative (total) activity changes represent an excellent indicator for activities of the corresponding genes. It is not only the quality of enzymes that changes, the profile of isoenzymes changes as well (qualitative changes), which leads to the development of a specific tumour metabolism.

Voluminous literature sources dealing with isoenzymes, collected over the recent years, confirm that if not all then the majority of enzymes have their own isoenzymes. The existence of diverse molecular forms of a single enzyme with different kinetics has its biological purpose, which is to ensure metabolic functions of certain tissues and organs as well as alternations of these functions in various physiological and pathological conditions.

Certain enzymes and isoenzymes are often used as markers in diagnosing malignant diseases. The activity of many enzymes is altered in malignant diseases. It is highly probable that the presence or a change in the activity of certain enzymes and their isoenzymes is an indicator of the presence of a malignant tumour. Although these enzymes represent products of malignant cells, they are still not strictly specific for malignity because they can sometimes appear in some other diseases.

Besides changes in the total enzyme activity, there are characteristic profiles of tumour type isoenzymes. According to the current attitude, it is much more important, specific, and sensitive to determine the isoenzyme activity than to measure the total enzyme activity since extensive changes in the composition of enzymes are often associated with a somewhat altered total enzyme activity.

Further research is necessary to standardize methods, choices and procedures as well as detailed laboratory and diagnostic correlations in order to show whether isoenzymes can be used with a high level of confidence to identify both patients having hidden metastatic changes and premalignant and early malignant

**Abbreviations**

LDH	– lactate dehydrogenase
HK	– hexokinase
PK	– pyruvate kinase
PK-K <sub>4</sub>	– pyruvate kinase 4-isoenzyme
EGTA	– ethylene glycol-bis (beta-aminoethyl ether NN – tetraacetic acid)

lesions before pathohistological changes become evident.

Although various glycolytic enzymes and their isoenzymes cannot be regarded as strictly specific tumour markers, they may contribute to better diagnostics of malignant tumours besides other laboratory tests. The activity of glycolytic enzymes in malignant tumours, particularly lactate dehydrogenase (LDH), (EC.1.1.1.27), hexokinase (HK) (EC.2.7.1.1.), pyruvate kinase (PK) (EC.2.7.1.40.) and their isoenzymes have been studied by many authors [13-15] because of the enormously increased aerobic and anaerobic glycolysis, which is one of the dominant characteristics of malignant neoplasms.

This research was aimed at performing the quantitative and qualitative analysis of lactate dehydrogenase, pyruvate kinase and hexokinase and breast cancers and dysplasia and at assessing possibilities of enzymatic diagnostics of these malignant tumours. The reliability of the applied methods was tested for the same purpose.

**Material and Methods***Origin and Source of the Material*

The material obtained from the patients operated on at the Institute for Oncology of the Faculty of Medicine in Sremska Kamenica included 60 samples of breast cancers, 20 samples of benign breast tumours and 40 samples of 1<sup>st</sup> and 2<sup>nd</sup> degree dysplasia of the breast. These patients had not been treated by either radiotherapy or cytostatics before the surgical intervention and each sample was previously verified patho-histologically. Blood serum was obtained from healthy blood donors, and the liver and heart of healthy Wistar male rats, aged 2-3 months, weighing about 200 gr, were used as the biological material. The experimental animals were obtained from the experimental animal farm of the Institute for Biochemistry of the Medical Faculty in Novi Sad.

*Preparation of Experimental Animal Tissue Extract*

The extracted organs (liver and heart) were washed several times with the saccharose medium containing 250 mmol/l sucrose, 10mmol/l of Tris-hydrochlorid acid (HCl), 5 mmol/l of EGTA, pH7.4. The tissue cut into small pieces was homogenized in the glass homogenizer – it took six movements of the piston from top to bottom to break up the liver cells and about 20 of them for the heart. The homogenate was poured into the test tube and centrifuged at 2000 rpm for 10 min, i.e. 10000 rpm for 20 min. The supernatant was decanted and designated. The proteins were determined in each supernatant by Biuret method [16]. The supernatants obtained

in this way were used to check the applied methods, i.e. they were the internal control of the experiment performed.

*Serum Preparation*

Blood was obtained from voluntary donors by the cubital vein puncture. Serum was separated from the same blood sample after coagulation.

*Preparation of Human Malignant Material for Enzyme Analyses*

The material (breast cancers, malignant tumours, and dysplasia) was collected during the surgeries and kept at -20° C in hypotonic buffer containing 50.00mmol/l of sodium chloride (NaCl), 5 mmol/l of phosphate buffer potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) pH 7.20 before being assayed. The frozen breast tumour samples were cut into pieces by the microtome. Prior to extraction, the samples were thawed at room temperature. Breast cancers showed the highest heterogeneity due to a different relation between the stroma and malignant cells. In order to make the extraction of soluble proteins uniform, the tumour tissue was frozen and thawed three times; thus, better reproducibility was achieved in enzyme and isoenzyme extraction. Homogenization was done in the glass homogenizer very carefully and tenderly. Supernatants were obtained by differential centrifugation and they were used for measuring enzyme activity and proteins.

*Analyses of Enzyme and Isoenzyme Activity*

The vertical polyacrylamide gel disc electrophoresis according to the slightly modified Brewer and Ashworth's method was carried out by an apparatus designed by the company "Pleuger" Wijnegem-Belgium to separate LDH isoenzyme [17]. Having been separated and incubated, the gel was conserved in acetic acid (CH<sub>3</sub>COOH) and kept in dark for several years without a considerable loss of colour. The stained gels were analyzed and photographed. In some cases, the quantitative analysis of the photographed enzymograms was done as well by the Eppendorf densitometer and the relative isoenzyme values were expressed in percents. The total LDH activity was determined by the method based on the optical test according to Wroblewski et al [18] and the total hexokinase activity was determined by the method of Lamprescht et al [19]. The method of Gutmann and Bernt was used for measuring the total activity of PK [20] and pyruvate kinase-4-isoenzyme (PK-K<sub>4</sub>) was measured by the method, hereby enclosed, for the total PK activity with the addition of L-alanine.

The obtained results were statistically analyzed by determining the mean value (X) with the standard deviation (SD), and the comparison between the groups (dysplasia and cancer.) was done by Student's t-test. Sigma Stat 2.0 programme was applied for the statistical analysis, and the value p<0.001 was regarded as a statistically significant difference.

## Results

A great number of experiments were carried out to check the method of polyacrylamide gel disc electrophoresis and to find suitable experimental conditions for qualitative LDH isoenzyme determination in malignant tissues. The original method was modified. The duration of gel polymerization and electrophoresis varied as well as the amount of material. The analysis of human serum LDH isoenzyme and of rat liver and heart extracts was previously performed and the profiles obtained thereby were characteristic of the above biological samples. Such analyses represented the internal control of the method applied. LDH isoenzymes were designated from 1 to 5 as follows: the fastest migrating isoenzyme was designated LDH<sub>1</sub>-anodic and the slowest migrating one was designated LDH<sub>5</sub>-cathodic. The isoenzyme profiles of the control biological material are given on the original photographs. Fast isoenzymes prevail in blood serum of a healthy person. The greater part of LDH isoenzyme activity is found in the fast-moving fractions of LDH<sub>1</sub>, LDH<sub>2</sub> and LDH<sub>3</sub> (Figure 1). Such an enzyme profile is in accordance with confirmed findings of other authors [21]. LDH<sub>5</sub> fraction is dominant in the rat liver extract, whereas LDH<sub>5</sub> fraction is the most intense in the rat heart (figure 2 and 3).

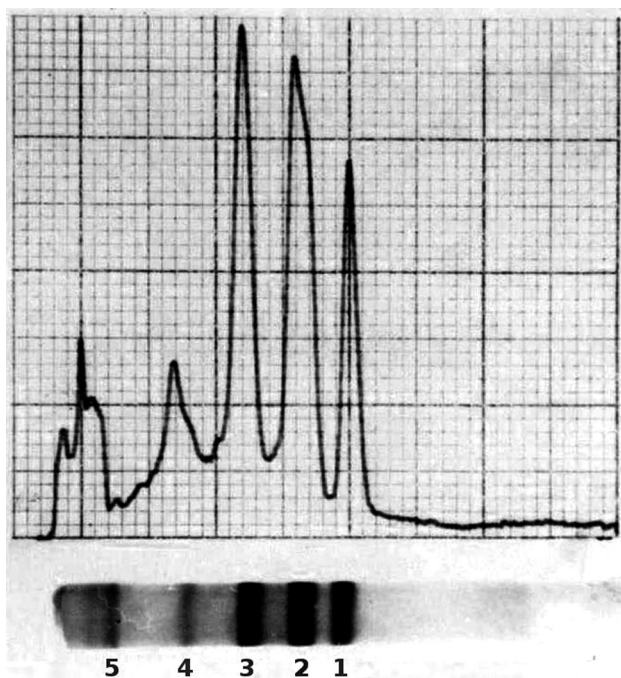


Fig 1. Densitometry of human serum LDH isoenzyme  
Slika 1. Dezintometrija izoenzima LDH humanog seruma

During the electrophoretic separation of enzymes of breast malignant and benign tissues and dysplasia, the most frequently used referral sample was a rat liver sample. This method was applied to analyze LDH isoenzyme in 120 samples of malignant and benign tumours of breast as well as 1<sup>st</sup> and 2<sup>nd</sup> de-

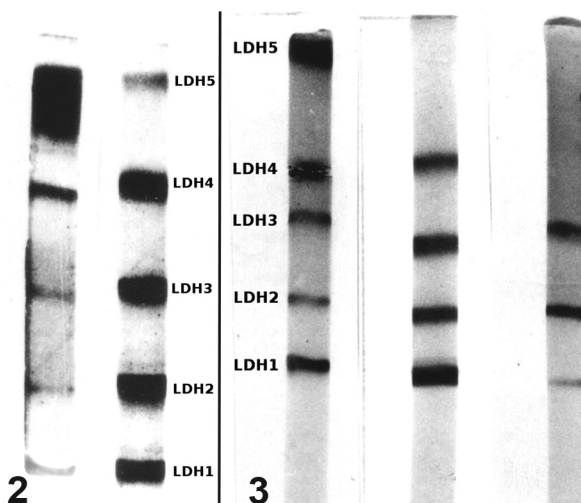


Fig. 2. Comparison of LDH isoenzyme profile - heart and liver of rat (from left to right)

Slika 2. Poređenje profila izoenzima (sleva nadesno) - jetra pacova, srce pacova

Fig. 3. Comparison of LDH isoenzyme profile obtained by parallel analysis - breast cancer, 2<sup>nd</sup> degree breast dysplasia, 1<sup>st</sup> degree breast dysplasia (from left to right).

Slika 3. Poređenje profila izoenzima LDH dobijenih na osnovu paralelne analize (sleva nadesno), karcinom dojke, displazija dojke II stepena, displazija dojke I stepena

gree dysplasia. The original photographs of the obtained enzymograms are shown in the Figure 3 and 4. Some significant results were obtained by the parallel analysis of the samples of breast cancer, tumour and dysplasia on the same electrophoretic gel. Not a single breast dysplasia sample was found to have LDH<sub>5</sub> enzyme fraction, whereas this molecular form appeared in 88% of breast cancer samples. The obtained results point to the increased LDH<sub>3</sub> and LDH<sub>4</sub> fraction in benign breast tumours and the absence of LDH<sub>5</sub> fraction (Figure 4).

In some samples, LDH isoenzymes were determined quantitatively by photographing stained gels using Eppendorf densitometer at wavelength of 500nm (Figure 1). The relative ratio of LDH isoenzyme fractions in human serum, rat liver and breast cancers and dysplasia was determined according to the densitographic enzymogram (Table 1).

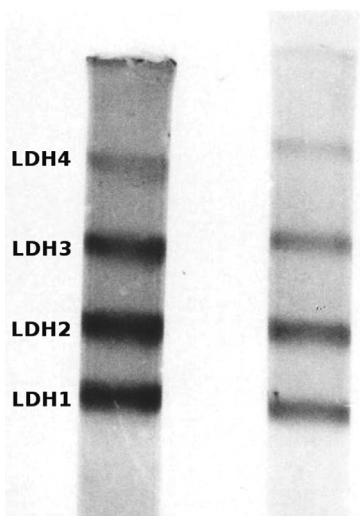
In human serum, the maximum of activity was observed in LDH<sub>1</sub>, LDH<sub>2</sub>, LDH<sub>3</sub> fractions. The amount of quantity of LDH<sub>5</sub> fraction detected in the rat liver extract was 73%. The increase in LDH<sub>5</sub> fraction was significant in breast cancer samples and it was 48% of activity of all enzyme fractions, whereas this fraction was represented in breast dysplasia by only 2% of the total activity.

The authors of this study measured the activity of hexokinase in the samples of breast cancers and dysplasia and the obtained results are shown in Table 2, indicating that there was a significant increase in all tested malignant tissues. The activity of hexokinase was three times higher in breast cancer than in breast dysplasia.

**Table 1.** The relative ratio of LDH isoenzyme in human serum, rat liver, breast cancer and breast dysplasia

**Tabela 1.** Relativan odnos izoenzima LDH u humanom serumu, jetri pacova, karcinomu dojke i displaziji dojke

Isoenzyme Izoenzimi	Human serum Humani serum (%)	Rat liver Jetra pacova (%)	Breast cancer Karcinom dojke (%)	Breast dysplasia Displazija dojke (%)
LDH <sub>1</sub>	22	5	8	18
LDH <sub>2</sub>	33	4	12	19
LDH <sub>3</sub>	21	4	13	50
LDH <sub>4</sub>	17	14	16	18
LDH <sub>5</sub>	7	73	48	2



**Fig 4.** Isoenzyme profile of LDH in benign breast tumour  
**Slika 4.** Izoenzimski profil LDH benignih tumora dojke

In addition to the two mentioned enzymes, which belong to the glycolytic pathway, the authors also measured the total activity of pyruvate kinase and K<sub>4</sub> isoenzyme in the samples including breast malignant tumours and dysplasia. The results, given in Table 2, show that the enzyme was about ten times higher in breast cancers than in breast dysplasia. K<sub>4</sub> isoenzyme was also measured in all tested samples in the presence of L-alanine and the obtained results are given in Table 2 as well. The analysis of PK-K<sub>4</sub> activity shows that the increase in the total pyruvate kinase activity in malignant tissues mainly results

**Table 2.** Enzyme activity in breast cancer and breast dysplasia

**Tabela 2.** Enzimaska aktivnost karcinoma dojke i displazija dojke

Enzyme Enzimi	Enzyme activity (nmol/min mg of protein) Enzimaska aktivnost (nmol/min mg proteina)	
	Breast dysplasia Displazija dojke	Breast cancer Karcinom dojke
Pyruvate kinase/Piruvat kinaza	7.50 ± 0.80	78.40 ± 11.62
PK-K <sub>4</sub>		48.30 ± 7.98
% of other activities % preostale aktivnosti		38.40 ± 0.34
Hexokinase/Heksokinaza	2.10 ± 0.26	6.20 ± 0.73

X ± SD; p < 0.001

SD – standard deviation/standardna devijacija; p – value/vrednost; statistically significant correlation at the level of p < 0.001/statistički značajna korelacija na nivou p < 0,001

from the increased K<sub>4</sub> isoenzyme activity, which is about fifty times higher in breast cancers than in breast dysplasia (Table 2).

### Discussion

The application of enzyme and isoenzyme analysis has become widespread in diagnostics of malignant diseases and follow-up of therapy effects. Regardless of the cause and mechanism of malignant transformation and progression of malignant neoplasm, one of the essential characteristics of tumours is a deep change in the protein synthesis programme, which results in the synthesis of proteins unusual for the type of cells from both the qualitative (isoenzymes) and quantitative (the total enzyme activity) aspect.

It was assumed that the planned biochemical analyses aimed at identifying changes in enzyme activity and isoenzyme profiles (lactate dehydrogenase, hexokinase, pyruvate kinase) in malignant tumours would offer significant possibilities for early diagnosis of malignomas, since these methods are sensitive and specific. Lactate dehydrogenase isoenzyme was analyzed by polyacrylamide gel disc electrophoresis method. The organ extracts of experimental animals (liver and heart), as well as human serum, were used to introduce and check the parameters of the applied method. Our analyses have shown that a high percentage of breast cancer (88%) is associated with the appearance of LDH<sub>5</sub> isoenzyme, whereas this enzyme has been found neither in even a single benign tumour of breast nor in samples of breast dysplasia. The most valuable result obtained by this study is the change of LDH isoenzyme profile in malignant tumours in the sense of reduced LDH<sub>1</sub> and the appearance of LDH<sub>5</sub> isoenzyme. The appearance of LDH<sub>5</sub> fraction, which is characteristic of breast malignant alteration, may be very important in diagnostics and differential diagnostics regarding benign tumours and dysplasia of the breast. The method of polyacrylamide gel disc electrophoresis, which is sensitive and simple, has shown that 88% of malignant tumours are associated with the appearance of LDH<sub>5</sub> isoenzyme. According to the experience, the technique of polyacrylamide gel disc electrophoresis detects subtle changes in malignant tissues and offers great possibilities in diagnostics of malignant diseases. The method itself and the obtained results can be repeated and checked with high reliability. Such an LDH isoenzyme profile in malignant tumours is in accordance with findings of other authors [22,23]. More recent research [24] has highlighted the great importance of determining the total activity of LDH and isoenzyme profile in diagnostics and treatment of patients with testicular cancer. The total LDH activity and isoenzyme profile were measured in patients with breast cancer [25]. The authors have pointed to the elevation of LDH<sub>5</sub> isoenzyme and associated the increased activity of this enzyme with short survival of the patients.

The results of measuring the activity of hexokinase in malignant tumours and dysplasia of breast suggest that there is a correlation between malignant process and increased activity of this enzyme. Our results have been compared with those from literature [26]. The hexokinase activity was measured in the tissues of patients having colon cancer, whereby a great difference was also confirmed in the activity of enzymes in malignant tissues as compared to surrounding normal tissues, which were not affected by malignant process. Our results of measuring the total hexokinase activity in human cancers are in accordance with those of other authors [27], who have claimed that the hexokinase activity is much higher in breast cancers than in normal tissues of the breast.

The activity of pyruvate kinase, being one of the key enzymes of glycolysis, is elevated in malignant tumours, probably resulting from an increased energy demand of malignant tissues [28]. A great difference was found when measuring the total pyruvate kinase activity in breast cancer as compared with the values measured in breast dysplasia. This increase was about 10 times higher in breast cancer than in breast dysplasia. These data may tell us whether the change is malignant or benign, since  $K_4$  isoenzyme [29] represents a specific indicator of malignant alteration. According to the obtained results, the more malignant the tumour, the higher the percentage of inhibition with L-alanine; therefore, the percentage of inhibition points to the level of malignity. The test of L-alanine inhibition can be done in 10 minutes and thus, in a very short time, data can be obtained on the malignant potential of tumour. The obtained results are in accordance with data from literature [30]. The more recent research included measuring the presence of  $K_4$  isoenzyme in different malignant tissues (lungs, breast, brain). Using allosteric inhibition of  $K_4$  isoenzyme with L-alanine, the same authors have proved that the

percentage of inhibition exceeding 20% is significant for diagnostic purposes. According to the same authors, tumours showing inhibition over 80% belong to a group of very malignant tumours.

### Conclusion

The planned research was important for the application of enzyme diagnostics of malignant tumours in our conditions, which should help in those cases when there is a dilemma whether the changes are malignant or benign. Morphological analyses can detect only advanced changes. Our assumption was that biochemical analyses of changes of enzyme activity and isoenzyme profile could offer greater possibilities for early diagnosis of breast cancer.

The study included qualitative and quantitative changes in the activity of lactate dehydrogenase, pyruvate kinase and hexokinase. According to the obtained results, the following conclusions can be made: the development of activity of lactate dehydrogenase (5.-isoenzyme) represents a very important characteristic of the tested cancers where this fraction was present in 88% of the analyzed cancer samples; lactate dehydrogenase (5.-isoenzyme) was not detected in breast dysplasia and benign tumours; the obtained data suggest that the malignant process is associated with the altered profile of lactate dehydrogenase isoenzyme, meaning that lactate dehydrogenase (5.-isoenzyme) appeared; an increased activity of hexokinase enzyme was detected in all breast cancer samples.

The activity of pyruvate kinase (4.-isoenzyme) was 50 times higher in breast cancer than in breast dysplasia. Measuring the activity of this isoenzyme represents a considerably more specific and more sensitive parameter of malignant alteration than measuring the total activity of pyruvate kinase.

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### Sažetak

#### Uvod

Istraživanja su imala za cilj da se ispita ukupna aktivnost enzima i profil izoenzima karcinoma i displazija dojke na humanom materijalu, kao i da se procene vrednosti podataka s aspekta poboljšanja dijagnostike.

#### Materijal i metode

Ispitivana je aktivnost laktat dehidrogenaze i profila njenih izoenzima, piruvat kinaze i heksokinaze. Ispitivanje je izvedeno na 60 uzoraka karcinoma dojke, 20 uzoraka benignih tumora dojke, 40 uzoraka displazija dojke I i II stepena kod pacijenata operisanih u Institutu za onkologiju Medicinskog fakulteta u Sremskoj Kamenici. Za separaciju izoenzima laktat dehidrogenaze primenjena je vertikalna disk elektroforeza na polijakrilamidnom gelu, po metodi Brewera i Ashwortha, uz manje modifikacije.

**Ključne reči:** Karcinom dojke; Displazija dojke; Biološki markeri; Laktat dehidrogenaza; Piruvat kinaza; Heksokinaza; Dijagnoza; Rana detekcija karcinoma

Rad je primljen 12. V 2011.

Prihvaćen za štampu 15. V 2011.

BIBLID.0025-8105:(2012);LXV:5-6:200-205.

Aktivnost svih ispitivanih enzima merena je u uslovima linearne kinetike u funkciji vremena i koncentracije enzima.

#### Rezultati i diskusija

U 88% analiziranih uzoraka karcinoma dojke nađena je laktat dehidrogenaza (5-izoenzim), dok u displazijama dojke ovaj enzim nije uočen. Piruvat kinaza (4-izoenzim) u karcinomu dojke povećana je oko 50 puta u odnosu na displazije. Aktivnost heksokinaze u karcinomu dojke triput je veća od displazije dojke. Laktat dehidrogenaza (5-izoenzim) i piruvat kinaza (4-izoenzim) predstavljaju posebno bitne i pouzdane markere maligniteta. Dobijeni rezultati kvantitativnih i kvalitativnih promena u aktivnosti enzima mogu se koristiti za poboljšanje dijagnostike i rane dijagnostike maligne neoplazme dojke.