Determination of Acid β -Galactosidase Activity: Methodology and Perspectives

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Abstract Early, accurate diagnosis of lysosomal storage disorders is a major challenge, even for trained specialists. Finding innovative, accurate diagnostic methods, and high throughput, cost-effective tools are crucial to medical progress and will contribute to improved quality of life. The goal of this work was to improve currently used protocols to determine activity of acid β -galactosidase, and discuss the possibility analysing lysosomal enzymes with microfluidic systems. A principle of the determination of β -galactosidase activity was fluorometric measurement of a deprotonated form of 4-methylumbelliferone released in the enzymatic reaction. Measurements were performed using Jurkat T cells as a source of the enzyme. We observed the temperature-dependent substrate inhibition effect and determined the substrate (4-MU- β -D-galactopyranoside) concentration which should be used to determine acid β -galactosidase activity at 37 °C (0.8 mM) and at room temperature (0.6 mM). We proved that the sample incubation time may be significantly reduced to only a few minutes. We also showed that the amount of alkaline buffer used to stop the enzymatic reaction may be minimized and even, in some cases, eliminated. The presented results show how the sensitivity of the available methods to diagnose patients suffer from gangliosidosis GM1 or Morquio B disease can be improved. The proposed method may be easily implemented with microfluidic systems, which currently are promising tools for point-of-care applications.

Keywords β -Galactosidase activity · Lysosomal storage disorders · Substrate inhibition · Point of care · Microfluidic device · Lab on a chip

Introduction

Acid β -galactosidase (EC 3.2.1.23) (GLB) is a lysosomal hydrolase that may be defective with respect to gangliosides, lactosylceramide, asialofetuin and oligosaccharides [1]. Deficiency of this enzyme results in accumulation of GM1 ganglioside leading to GM1 gangliosidosis, or keratan sulfate leading to Morquio B disease [2]. GM1 gangliosidosis and Morquio B disease are rare, progressive lysosomal storage disorders (LSDs) that have devastating impact on the patient and family. Most patients are clinically normal at birth, but the symptoms appear quickly in childhood [3]. If untreated, severely affected patients often die in the first decade of their life. Currently, patients with β -galactosidase deficiency can only benefit from symptomatic treatments, but active investigations into enzyme replacement, substrate deprivation, chemical chaperon, and gene therapies are ongoing [4, 5].

While some therapies have great potential, their efficacy will rely heavily upon the early and accurate diagnosis of the disorder, ideally, before neurologic symptoms arise [3]. There are multiple approaches to the diagnosis of LSDs patients: histological examinations, identification of storage material in the patient's tissues or in urine, DNA-mutation analysis, and determination of enzyme activity [6]. However, all of them have some limitations and are not suitable for early diagnosis. Histological examinations and identification of storage material are rather used to confirm the diagnosis, when symptoms are visible and obvious. DNA-mutation analysis is time-consuming and, because of

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a large number of mutations of genes coding enzymes (e.g. over 100 mutations distributed along the β -galactosidase enzyme [4]), cannot be used as a high throughput method for screening tests.

Finally, analysis of enzyme activity is the most used diagnostic method by many laboratories in the world. Biochemical diagnosis of GM1 gangliosidosis, Morquio B disease and most other lysosomal storage disorders is based on determination of activity of a proper enzyme using usually a derivative of 4-methylumbelliferone (4-MU) as a substrate [7] and leukocytes or fibroblasts from a patient [8]. The principle of the method of biochemical diagnosis in each case is fluorometric measurement of a deprotonated form of 4-MU released in the enzymatic reaction. The main steps of a diagnostic procedure are: (i) enzyme release by cell lysis [9], (ii) sample incubation with substrate and other necessary reagents such as inhibitors of isoenzymes, (iii) termination of enzymatic reaction with alkaline buffer, and (iv) measurement of fluorescence. The method is suitable for screening and results in a complete differentiation between affected individuals and controls. However, the method is still not unambiguous in case of heterozygotes. There are still too many false-positive and false-negative test results. The reliability and accuracy of the methods used to determine the activities of lysosomal enzymes and classify patients for a proper therapeutic group need urgent improvement.

Recently, performing enzyme assays using dried blood spots (DBS), often in combination with tandem mass spectrometry, has become popular [8, 10, 11]. The use of DBS offers such advantages as: relatively painless and noninvasive sample collection; easy handling, transport and storage of a sample; and low cost [12]. However, because of the very small amount of blood collected, long sample incubation time is required, and subtle differences between low enzyme activity and no activity can be lost [7]. Moreover, tandem mass spectrometry is still limited to a few reference laboratories worldwide [8].

To our knowledge, there are no miniaturized devices, high throughput (HTS) or point of care (POC) microsystems dedicated to early and fast diagnosis of GM1 gangliosidosis, Morquio B disease and other lysosomal storage disorders. Miniaturized devices known as lab-on-a-chip (or micrototal analysis systems) could meet current needs and became a powerful tool for diagnosis of LSDs. Using microfluidic systems to determine the activity of lysosomal enzymes could have many advantages. Many steps of an analysis such as cell lysis, reagents' mixing, incubation or detection could be performed within one device. Compared to currently used protocols, microsystems may provide a significant reduction of the analysis time. Moreover, they could minimize reagents and energy consumption and enable to monitoring of each step of analysis. Due to the possibility of integration with

commercially available detectors, they hold much promise for point-of-care applications. Many already developed enzyme-based bioanalytical microsystems have several distinct advantages which include real-time operation, high sensitivity and specificity, easy operation, portability, cost-effectiveness, and the possibility for mass production [13–15].

In the work discussed in this paper, we looked closely at the steps of currently used diagnostic protocols, verified them and tried to identify potential improvements. The kinetics of the reaction catalyzed by β -galactosidase at different temperatures, sample incubation time, effect of the amount of alkaline buffer on the final pH of a reagent mixture and the need to add this buffer were studied. We also discussed the use of current diagnostic protocols and how these protocols could be made suitable for implementation within microfluidic chips.

Materials and Methods

Cells

Jurkat cells, a human T-cell line, were used as a source of enzyme in our investigations. The culture medium for routine culture contained: 88.9 %_{vol} RPMI-1640 medium, 10 %_{vol} fetal bovine serum (FBS), 1 %_{vol} 25 mM L-glutamine and 0.1 %_{vol} 100 mM penicillin and streptomycin.

The cells were cultured in a CO₂ cell culture incubator (HERAcell 150, Thermo Scientific) at 37 °C and in a 5 % CO₂ atmosphere. In order to prepare cell suspension, the cells were centrifuged and suspended in phosphate buffered saline buffer (PBS). The density of cell suspension was determined precisely using an automated cell counter (Countness, Invitrogen). All reagents to cell culture were purchased from Sigma Aldrich (Poznan, Poland).

Cell Lysis Buffer

In order to release the enzyme, cells were lysed with non-denaturing cell lysis buffer containing 10 mM imidazole (Fluka), 0.5 M sodium chloride (Sigma Aldrich), 1 % Triton X-100 (Sigma Aldrich), 0.2 mM sodium *ortho*-vanadate (Sigma Aldrich) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF, Fluka) dissolved in 100 mL of DI water.

Chemicals

4-Methylumbelliferone (4-MU), 4-MU- β -D-galactopyranoside, albumin, glycine, citric acid, sodium phosphate dibasic, sodium chloride and sodium hydroxide were purchased from Sigma Aldrich (Poznan, Poland).

Enzyme Assay

The protocol to determine β -galactosidase activity, which was verified in our investigations, was developed based on Chamoles et al. [16], Civallero et al. [8], Gasparotto et al. [2] and a diagnostic protocol used by the Institute of Psychiatry and Neurology in Warsaw, Poland. All of these were based on protocols developed 30–40 years ago.

The enzymatic reaction was initiated by mixing: (i) 250 μ L lysed cell suspension with 0.1 % albumin (220 μ L cell suspension in PBS lysed immediately after adding 30 μ L cell lysis buffer) and (ii) 750 μ L 4-MU- β -D-galactopyranoside in 0.05 M citrate–phosphate buffer, pH 4.5 with 0.45 % NaCl. The final substrate concentration in the reaction mixture was 0.8 mM. The mixture was incubated at 37 °C for 30 min. After this time the enzymatic reaction was terminated by adding 10 mL 0.1 M glycine–NaOH buffer, pH 10.7. The same protocol was used for the blank mixture. The relative fluorescence (excitation 365 nm, emission 445 nm) was measured using a spectrofluorimeter (FluoroMax-3, Jobin–Yvon Inc.). The results were compared with a 4-MU standard curve. The enzymatic activity was expressed as μ U per number of cells (the number of used to the enzymatic reaction cells recalculated to 10^6), where U is the amount of enzyme that catalyzes the conversion of 1 micromole of substrate per 1 min.

Study of Reaction Kinetics

In order to investigate the kinetics of the reaction catalyzed by acid β -galactosidase, the reaction velocity versus different substrate concentration was studied. Measurements, according to the given protocol, were performed at following final concentrations of 4-MU- β -D-galactopyranoside: 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mM. The investigations were performed at 3 temperatures: 17, 25 and 37 °C.

Analysis of Incubation Time

β -Galactosidase activity was measured according to the given protocol after different incubation times at 25 and 37 °C. Measurements were carried out after 2, 5, 10 and 30 min of incubation.

Termination of Reaction with Alkaline Buffer

1 mL portions of 0.1 M glycine–NaOH buffer, pH 10.7 were added to 1 mL 0.05 M citrate–phosphate buffer, pH 4.5. Changes in pH of the buffer mixtures after adding each 1 mL of alkaline buffer were recorded. The fluorescence intensities of 4-MU at a concentration of 0.5 μ M in the different pH buffer mixtures were also measured.

Statistics

Statistical analyses were performed using the Microsoft Office Excel 2003 software. Values of the enzyme activity are expressed as mean $\pm$ SD ($n = 5$).

Results

Kinetics of Acid β -Galactosidase

Results of our investigations on acid β -galactosidase kinetics are presented in Fig. 1. At 37 °C the kinetics of β -galactosidase is described by the Michaelis–Menten model, which confirms the literature reports. In this case, the enzyme activity should be determined for a saturating substrate concentration range, when the reaction rate reaches its maximum potential. Thus, the activity of acid β -galactosidase at 37 °C should be determined for a substrate concentration at least of 0.8 mM.

At 17 and 25 °C the substrate inhibition effect is observed [17]. Nonhyperbolic behavior is caused by the formation of an inactive substrate–enzyme–substrate complex [18]. At both temperatures, the maximum velocity of the enzymatic reaction occurs for 0.6 mM of 4-MU- β -D-galactopyranoside, and it is the concentration which should be used to determine acid β -galactosidase activity at temperatures lower than physiological one.

Incubation Time

Values of acid β -galactosidase activity obtained after different incubation times at two temperatures are detailed in Table 1. According to our results (see “Results”), β -galactosidase activity at 25 °C was determined for a substrate concentration of 0.6 mM, whereas at 37 °C for 0.8 mM. All measurements were highly repeatable (SD < 10 %).

The obtained values of β -galactosidase activity after 2, 5, 10 and 30 min of incubation are very similar. There is a linear relationship between the amount of released 4-MU and time.

Termination of Enzymatic Reaction

The results of the amount of glycine–NaOH buffer used to change the pH of the reaction mixture are presented in Fig. 2. The fluorescence intensities of 4-MU in the obtained different pH buffer mixtures were also measured (see Fig. 3). The results show that sixfold dilution of the reaction mixture with alkaline buffer provides a pH (9.5) that varies slightly when adding the next portions of this buffer. The fluorescence intensity of 4-MU also does not change significantly above a pH of 9.5.

Fig. 1 Velocity curves for acid β -galactosidase as a function of 4-MU- β -D-galactopyranoside concentration ranging from 0.01 to 1 mM at: **a** 37°C (Michaelis–Menten kinetics), **b** 25°C (substrate inhibition kinetics) and **c** 17°C (substrate inhibition kinetics)

Discussion

The presented results of the investigations on the enzyme kinetics show that the determination of acid β -galactosidase activity can be performed at a wide range of temperatures, including room temperature. The ability to measure acid β -galactosidase activity at room temperature is especially important when designing new point of care (POC) microsystems. In this way, the structure of such systems is less complicated. They do not have to contain an additional, costly thermostating module with its own power, which

makes these systems cheaper, portable and able to be mass-produced. However, in order to validate such microsystems and obtain repeatable and reliable test results, measurements should be carried out in air-conditioned rooms with constant temperature.

According to the results of the optimization of the sample incubation time, the total time of analysis may be significantly reduced to only a few minutes. However, in some cases, such as when using an extremely small amount of biological material from a patient (blood, cultured fibroblasts) or when a sensitive spectrofluorimeter is not available, longer incubation times are inevitable. In other cases, shortening the incubation time should be considered.

In this work we also studied the amount of glycine–NaOH buffer used to change the pH of the reaction mixture. Addition of an alkaline buffer to the incubated mixture (pH 4.5) leads to inactivation of the enzyme and, as a result, terminates the enzymatic reaction. Moreover, at alkaline pH the product of the enzymatic reaction (4-MU) is highly fluorescent [7], much more than its protonated form (excitation 320 nm, emission 445 nm).

Most of the protocols suggest determining the fluorescence intensity of 4-MU at a pH above 10 [7], but efforts to further increase a pH of the mixture lead to sample dilution and consequently tend to reduce the sensitivity of the method. Future studies on the concentration and pH of buffers used to terminate the enzymatic reaction could minimize the determined amount of alkaline buffer even more. It could help to improve the sensitivity of the current methods. Moreover, reducing the amount of alkaline buffer would make it possible to shorten the sample incubation time.

There is also a possibility to determine a protonated form of 4-MU directly in the reaction mixture. This is done by omitting the sample dilution step. To stop a reaction, an enzyme can be denaturated by heating. Unfortunately, in this case it is very difficult to control the time of an enzymatic reaction, which is necessary to determine an enzyme activity. Moreover, high temperature leads to spontaneous hydrolysis of a substrate.

Another option to determine a protonated form of 4-MU is a flow analysis using e.g. microfluidic systems. In the flow analysis, enzyme inactivation is not required. By changing the flow rates of the (i) fibroblast or leucocyte suspension and (ii) the other reagents introduced to a microsystem, while maintaining a constant ratio, the concentration of the released product after different reaction times can be determined [9].

The next option, without enzyme inactivation and sample dilution, is a stopped-flow analysis. This may also be done within a microfluidic chip. Then, after introduction to the system all reagents and their mixing, the flow is stopped and the increase of the product's fluorescence in

Table 1 Values of acid β -galactosidase activity after different incubation times at 25 and 37 °C

Temperature (°C)	Incubation time (min)	Enzyme activity ($\mu\text{U}/10^6$ cells) (mean $\pm$ SD, $n = 5$)
25	2	187.8 $\pm$ 14.7
	5	192.2 $\pm$ 16.8
	10	186.8 $\pm$ 16.6
	30	198.4 $\pm$ 15.6
37	2	437.8 $\pm$ 37.6
	5	433.4 $\pm$ 20.6
	10	441.6 $\pm$ 19.5
	30	455.2 $\pm$ 29.4

Fig. 2 Changes in pH of buffer mixtures after adding each 1 mL 0.1 M glycine-NaOH buffer, pH 10.7 buffer to 1 mL 0.05 M citrate-phosphate buffer, pH 4.5 **Fig. 3** Fluorescence intensities of 0.5 μM 4-MU in the different pH mixtures of 0.05 M citrate-phosphate buffer, pH 4.5 and 0.1 M glycine-NaOH buffer, pH 10.7

time can be measured. The slope of the obtained curve is used to determine the enzyme activity. This method can also be performed in a standard cuvette, but then all the previous steps of the analysis must be done manually and there is a need to use more reagents.

Conclusions

In the work discussed in this paper, we present ways to improve the sensitivity of the available methods to diagnose patients suffering from gangliosidosis GM1, Morquio B disease and, because of similarities in diagnostic protocols, many other lysosomal storage disorders. We show that the determination of acid β -galactosidase activity can be performed at a wide range of temperatures, but due to the temperature-dependent substrate inhibition effect, substrate concentration should be selected precisely, according to the temperature. We also present that there is a linear relationship between the amount of released product (4-MU) and time, and the activity of β -galactosidase can be determined after a very short time of sample incubation. Moreover, we show that, depending on concentration and pH of a buffer used to terminate the enzymatic reaction, the required amount may be much smaller than the literature suggests, and, as a result, the sample is less diluted.

We also discuss the usefulness of microfluidic systems as point-of-care for lysosomal storage disorders diagnostics. In our opinion, the presented method, especially based on determination of a protonated form of 4-MU without enzyme inactivation, can be easily implemented with microsystems. After the validation of the analytical procedure, the properly developed microfluidic device could be ready for preliminary tests in clinical laboratories and become a powerful tool changing current diagnostic approach.

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