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On-line monitoring of enzymatic conversion of adenosine triphosphate to adenosine diphosphate by micellar electrokinetic chromatography

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Abstract

Capillary electrophoresis can be a valuable tool for the on-line monitoring of bioprocesses. The enzymatic conversion of nucleotide adenosine triphosphate (ATP) to adenosine diphosphate (ADP) by hexokinase (HK) was monitored in the bioreactor interfaced by a laboratory-built microsampler to a capillary electrophoresis unit. The use of this specially designed sampling device enabled rapid consecutive injections to be performed without high-voltage (HV) interruptions. No additional sample preparation was required. The method of micellar electrokinetic chromatography, employing reversed electroosmotic flow (EOF) by cationic surfactant and reversed polarity mode provided a good resolution and short analysis time of less than 5 min. The samples were injected electrokinetically, using −25 kV voltage for 3 s and detected by their UV absorbance at 254 nm. The analytes were detected at a μg/ml level with a reproducibility of about 7%. To demonstrate the potential of CE in understanding the processes of biological interest, such as nucleotide degradation and metabolism, the investigation of the efficiency and the time course of the enzymatic transformation was carried out.

Keywords: Kinetic studies; Adenosine diphosphate; Adenosine triphosphate; Enzymes

1. Introduction

A fast development of biological science and technology has brought up the need for fast analysis methods, which enable to monitor bioprocesses constantly and provide rapid response. Enzyme-catalyzed reactions have a special significance in biology and biomedicine, therefore the investigation of such processes by analytical techniques is of prime importance. There have been several analytical methods developed during the recent years for the monitoring of enzymatic reaction kinetics. High-performance liquid chromatography based assays have gained an increasing popularity for these purposes [1–4]. Reversed-phase HPLC was applied by Nouri et al. for the investigation of enzymatic hydrolysis of wheat proteins [2]. Also the study of enzymatic hydrolysis of diperodon enantiomers in the blood serum by two-dimensional LC was recently described [3]. The on-line monitoring of enzymatic reaction, using LC with electrochemical detection and microdialysis sampling, was proposed by Torto et al. [4].

Mass spectrometric assay for the monitoring of enzymatic hydrolysis of penicillin has been developed by Ghassempour et al. [5]. Alternatively, enzyme kinetics was monitored by infrared spectroscopy in combination with chemometrics data processing [6–8] and by ^{1}H NMR spectroscopy [9]. Spectrofluorimetric assay for the continuous monitoring of medicinal esters has been also described in detail by Salvi et al. [10].

None of the methods mentioned above are ideal for the direct monitoring of the changes in the composition of the reaction medium. Some of them require the use of radioactive materials, which is neither economical nor environmentally friendly. Others require the reaction to be stopped by adding reagents into the system, making it inconvenient to establish a rapid kinetic analysis. For example, in most HPLC approaches for monitoring enzyme activity the sample preparation procedure is off-line, which adds a significant amount of work and leads to errors in analytical results. This requires a need for automated on-line monitoring systems with minimum/no sample preparation, especially if the rapid response is required due to, for example, process speed.

Recently, there appeared a remarkable interest in the use of CE for enzymatic reaction monitoring [11–13]. CE is a powerful analytical tool, ideally suited for that purpose. It is characterized by high-resolution separations,

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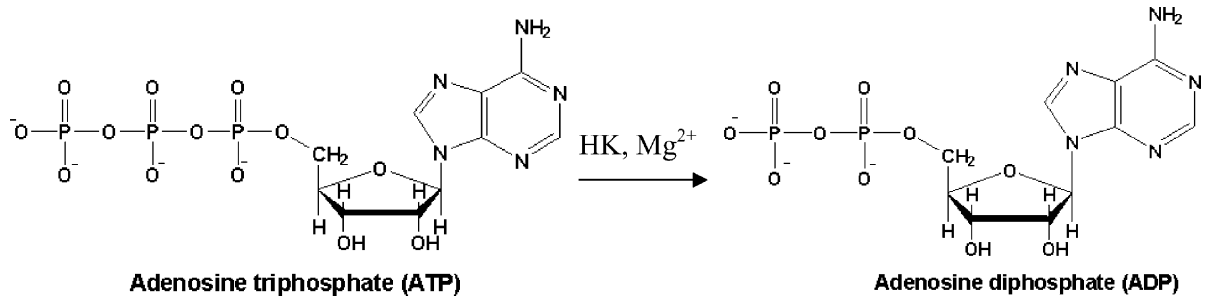

Fig. 1. Enzymatic hydrolysis of ATP to ADP by hexokinase (HK).

short analysis periods and a low sample load. The combination of these characteristics allows CE to monitor the enzyme reaction progress without disturbing the reaction. Capillary electrophoresis provides an efficient separation of the reaction products from the substrates in a short time, therefore it is possible to monitor both the loss of substrate and the formation of products simultaneously. Also CE takes aqueous samples directly from the reaction mixture without the need for stopping the reaction prior to analysis, which is very important for kinetic studies of rapid enzymatic processes. However, a fast CE analysis of reaction mixture requires specially designed CE equipment, perhaps even requiring the implementation of microchips [14], which is not commercially available at the moment. A possible solution for performing a rapid CE analysis of enzyme kinetics by using an ingenious but simple "short-end" injection has been suggested [15]. In another approaches, a in-capillary reaction procedure was applied for fast analysis of enzyme kinetics [16,17]. The latter technique requires a complex arrangement of reactant zones at the capillary inlet. The use of multiplexed capillary electrophoresis [18] for determination of protein kinase activity offers the potential of increasing the sample throughput, but at a price of increased instrumentation complexity.

The real restriction to the wide application of CE to enzymatic reaction monitoring seems to be manual sampling procedure, due to the fact, that most of the measurements have been performed off-line. This is in turn due to be the shortage of convenient on-line sampling/sample preparation devices. Although, commonly commercially available CE instruments are equipped with autosamplers, they are principally capable of performing on-line measurements. But, autosamplers, having a rigid operating program, are inflexible. The latter disadvantage has specially pointed out by the authors [11]. In this work, the autosampler runtime program has a fixed working sequence, which limited the speed of sampling.

Monitoring requires multiple computer-controlled samplings from the same reaction vessel, especially when the time resolution between experiments is an issue. The possibility of applying sophisticated sampling sequences of buffer, sample, and washing liquids at the capillary inlet could be advantageous in many cases of rapid kinetics measurements. For the sake of reproducibility, the sampling should be carried out without high-voltage (HV) interruption between consecutive injections. The sampler should also be able to operate with both small and large sample amounts. The samplers in commercially available instruments are suitable for introducing a large number of stationary samples, but are inconvenient for the introduction of samples, whose composition changes with time. Fang et al. [19] and Kuban and Karlberg [20,21] have provided possible prototypes for samplers for on-line monitoring applications, hyphenating the flow-injection analysis and CE. Dasgupta and co-workers have provided several innovative samplers for CE, capable of performing multiple injections of tiny amounts of sample from the same sample vessel [22,23]. The construction of the pneumatic autosampler, specially designed for multiple injection applications without HV interruptions, has been reported in [24]. This autosampler has been used for monitoring the production of organic acids by phosphate solubilizing bacteria [25], and for on-line analysis of biodegradation of phenols by *Rhodococcus* bacteria in a miniaturized bioreactor [26].

In this paper, we explore the possibilities of the pneumatic sampler further. We have developed an analytical method, using capillary electrophoresis for on-line monitoring of an enzymatic reaction, applying the pneumatic autosampler for multiple sample injections. The model reaction was chosen to be a well-known enzymatic conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) by hexokinase (HK). Hexokinase phosphorylates glucose to glucose-6-phosphate, using ATP as a substrate (Fig. 1). During this reaction ATP is converted to ADP. This catalytic process is thoroughly described in the literature and has been investigated by many methods. Thus, it was chosen as a good reference to estimate the characteristics of our monitoring equipment and procedure. We tested this reaction in various conditions, which allowed general estimation of the suitability of the developed CE method for monitoring of enzymatic reactions in general. First, the electrophoretic separation conditions were studied for an optimal monitoring of ATP hydrolysis. A separation method was developed that gave short analysis times and did not require sample pretreatment. Furthermore, a kinetic study of the hydrolysis

of ATP was carried out. Substrate and enzyme concentrations were varied and Michaelis–Menten constant was calculated.

2. Experimental

2.1. Equipment and procedures

The analytes were separated in a fused silica capillary column (Polymicro Technologies) of 40 cm (25 cm to detector) × 350 μm o.d. × 50 μm i.d. Prior to use, the capillary was flushed sequentially with 0.1 M NaOH for 5 min, deionized water for 5 min, and CE run buffer for 5 min. The CE run buffer consisted of 25 mM phosphate and cetyltrimethylammonium bromide (CTAB) at a concentration of 25 mM, buffer pH was 4.5. The electropherograms were recorded by means of an Isco CV^4 UV detector at 254 nm and transferred to a '486' personal computer memory via an "Adam" interface board (Advantech, Taiwan, Taipei, Taiwan) in a Labview environment (National Instruments, Austin, TX, USA). The separation was carried out at −25 kV.

The sample was introduced into the column using a laboratory-built pneumatic autosampler. The sampler has been described thoroughly in the publications [24,27] so only a brief description is given here. The schematic of the autosampler is shown in Fig. 2. The dimensions of the particular autosampler body used in this work are 4.7 cm × 1.6 cm × 1.6 cm and it was made of polyether ether ketone (PEEK) material. The sample and buffer reservoirs are connected to the sampler body with 100 mm × 0.7 mm PTFE tubing. The work of the sampler is based on the principle of rapid exchange of the buffer to the sample (and vice versa) in a narrow input channel (25 mm × 1.5 mm, 44 μl volume) into which the capillary and high-voltage electrodes are inserted. The flow of liquids is controlled

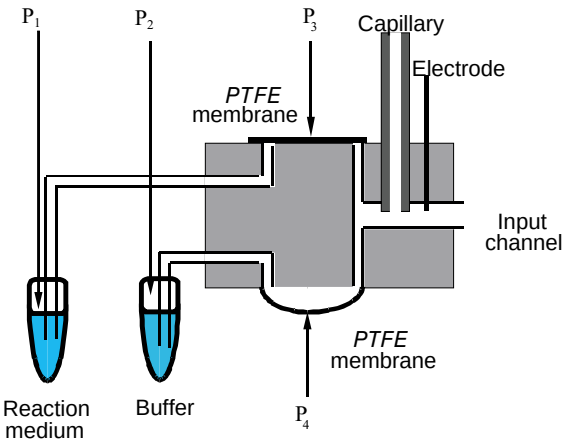

Fig. 2. Schematic of pneumatic autosampler. *P*1–*P*4 stand for pressures, while $P1 = P2 < P3 = P4$. During sample and buffer flow, either *P*3 or *P*4, is made equal to atmospheric pressure and the respective channel opens.

Table 1
Sampling sequence logic

Step	Action	*P*1	*P*2	*P*3	*P*4
1	Sample rinse	On	Off	Off	On
2	Electrokinetic sampling	Off	Off	On	On
3	Buffer rinse	Off	On	On	Off
4	Pherogram run	Off	Off	On	On

by a personal computer that activates the pair of solenoid valves (not shown in Fig. 2) connected to the compressed air tank thus providing pressure pulses necessary for the activating of the liquid flow. The applied pressure ranged between 0.3 and 0.8 × 10^5 Pa. The sampling logic is presented in Table 1. Channels are opened by PTFE films by liquid pressure and closed by air delivered through solenoid valves. Correct balancing of the liquid/air pressure is important and can be performed easily. It is evident from Table 1, that to execute the sampling process pressures must satisfy the following relationship $P1 = P2 < P3 = P4$.

The sampling mechanism is believed to be a mixed mode of both electrokinetics and hydrodynamics [27]. The contribution of a particular mode depends on timing parameters: if the buffer to the sample exchange is performed extremely rapidly, then the sample is introduced entirely electrokinetically and the sample amount depends only on the time during which the sample is kept still in the input channel. With increasing the time period when the sample is flowing through the inlet channel the hydrodynamic component of the sample introduction increases. The higher the pressure applied to the sample reservoir, the larger the amount of the sample introduced, and vice versa. The flexibility of the sampler construction allows operation with tiny amounts of sample (sub-milliliter range). This could be important if the available sample amounts are decreased. Another important feature of the sampler is that it always provides a fresh portion of the background electrolyte for the next injection, therefore eliminating changes of the buffer composition due to the electrolysis process.

The flow sampler has several properties, which can be considered advantageous as compared to the common samplers that appear in commercial instruments. First, the HV should not necessarily be interrupted during the sampling process. Although no special investigations have been performed with this particular sampler, this should improve the sampling process reproducibility since the voltage rise/fall during the sampling has been recognized to be the least reproducible part of the sampling procedure [28] Sampling relative standard deviation (R.S.D.) was estimated to be less than 1% [24]. No sample leakage or carry-over has been noticed. Second, flow samplers can be very easily computerized. Indeed, as demonstrated by us, sophisticated sample sequences can be generated easily [29]. Third, contrary to samplers included into commercial instruments, flow samplers are very useful for process monitoring. On-line sample preparation devices (such as membrane separators) can

easily be implemented into the flow line [25,26]. Forth, flow-sampling devices are very cheap and can be manufactured in lab by investigators themselves from the materials and parts available in every lab. This feature differentiates flow samplers advantageously from the CE devices manufactured on the silicone or polymeric substrate.

2.2. Chemicals

Nucleotides adenosine-5′-diphosphate and adenosine-5′-triphosphate were obtained from Sigma (Taufkirchen, Germany), hexokinase was from Calbiochem (Darmstadt, Germany). The sodium dihydrogenphosphate and cetyltrimethylammonium bromide for the CE buffer were purchased from Sigma. Sodium hydrogencarbonate, anhydrous D-glucose, magnesium sulfate and sodium hydroxide were obtained from YA-Kemia (Helsinki, Finland).

Water used for the buffer preparation was obtained from a Milli-Q-water system (Millipore, Bedford, MA, USA). All electrolytes and rinsing solutions were filtered before the use through 0.45 μm nitrocellulose Millipore filters.

2.3. Preparation of reaction media and monitoring procedure

The reaction medium was composed of 12 mM sodium dihydrogenphosphate solution (pH 7), containing 4.2 mM sodium hydrogencarbonate, 22 mM anhydrous D-glucose and 2.7 mM magnesium sulfate. Adenosine triphosphate was first dissolved in reaction buffer to obtain 100 mM stock solution. This was further diluted with the necessary amount of the same buffer, to obtain a reaction mixture volume of 100 ml. The initial concentration of ATP in the reaction mixture varied between 0.05 and 0.25 mM. The ATP hydrolysis was initiated by adding the hexokinase enzyme solution to the reaction cell. In this study three different enzyme concentrations (0.037, 0.05 and 0.068 U/ml) were analyzed. The enzymatic reaction medium was mixed continuously at 37 °C. After starting the experiment the reaction mixture was injected into the CE capillary by means of pneumatic autosampler every 2.5 min for approximately 30 min.

3. Results and discussion

3.1. CE method development

The hexokinase enzyme hydrolyses ATP to ADP, as shown in Fig. 1. First, for the separation of nucleotides a 25 mM phosphate buffer, pH 7.4, was chosen, according to the previously reported data [30,31]. A baseline separation of key compounds, using the common capillary zone electrophonesis (CZE) mode, is represented in Fig. 3a. To minimize the resolution time and to improve the separation efficiency, the method of micellar electrokinetic chromatography was applied for nucleotide analysis (Fig. 3b). For that, the cationic surfactant CTAB was added to background electrolyte, which causes the capillary wall to be positively charged and reversing the electroosmotic flow (EOF) toward the anode. Also the voltage polarity was reversed to re-establish the electroosmotic flow toward the detector. Buffer pH was reduced up to 4.5 for partial neutralization

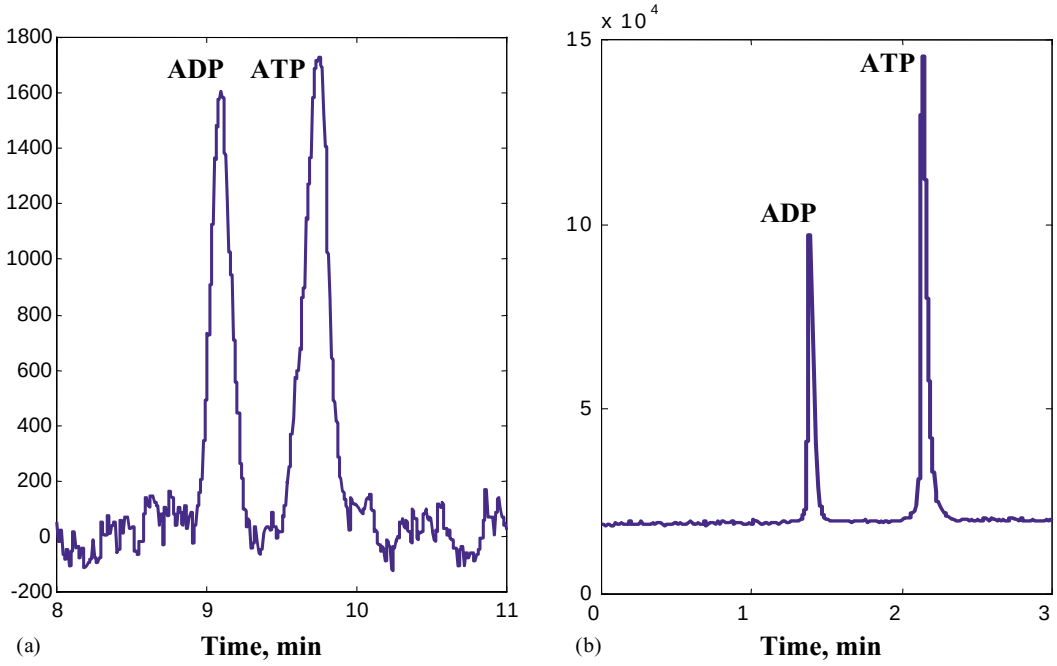

Fig. 3. Separation of the nucleotides by CE. Electrolyte: 25 mM NaH_2PO_4, fused silica capillary: effective length 25 cm × 50 μm i.d., UV detection at 254 nm. (a) Applied voltage +25 kV, buffer pH 7.5; (b) applied voltage −25 kV, buffer pH 4.5, 25 mM CTAB.

of nucleotides, which provided more complicated separation mechanism, when the nucleotide mobility depends on the charge to the radius ratio as well as on hydrophobic interactions with micelles. Despite the fact that the buffering capacity of the phosphate buffer is limited at that pH, electropherograms demonstrate that micellar electrokinetic chromatography (MEKC) employing reversed EOF provided a good resolution of ATP and ADP and short analysis time of less than 3 min.

The determination of nucleotides by CE was accomplished with UV detection at 254 nm. The other components of reaction mixture (glucose, glucose-6-phosphate, inorganic ions) have a low UV absorbance at this wave-length, thus allowing the detection of key compounds without a significant disturbance.

To evaluate the precision of CE system, an experiment was made, using replicate ($n = 3$) injections of ATP standard solution. The precision of migration times and peak areas was determined by calculating R.S.D. of measured parameters.

The migration times were precise with R.S.D. values within 1%, while the peak area reproducibility varied from 2.51 to 5.5% over the measured concentration range. The system gave a linear response to ATP concentration from 0.05 to 0.25 mM with a correlation coefficient of 0.977.

3.2. Enzyme assay and kinetics

In the second step, the determination of hexokinase activity was carried out. For that adenosine triphosphate was incubated with hexokinase in the presence of glucose and magnesium ions. The reaction was initiated by adding hexokinase. The automated sample injection device allowed monitoring of the enzymatic activity directly by using sample vial as catalysis reactor, so the determination of ATP enzymatic conversion kinetics was carried out without any reaction medium perturbation and sample preparation. We suppose that hexokinase separates within the first few seconds from the injection of the reaction mixture from the analytes band and reaction stops. The separation of ATP and ADP is shown in Fig. 4a. In Fig. 4b, a typical pherogram, obtained by repeated injection a reaction mixture of 0.037 U/ml, 0.1 mM ATP and buffer components is presented. Each group of peaks represents a new injection of the reaction mixture.

Time dependence of the conversion of ATP to ADP after the addition of hexokinase (0.037 U/ml) to a 0.1 mM solution of ATP is shown in Fig. 5. The reaction progression curves illustrate that both the formation of product and the disappearance of substrate can be monitored simultaneously and quantified according to their respective peak areas. During the course of the reaction a continuous decrease in substrate concentration was observed with an increase in product formation correspondingly, as one should expect.

3.3. Michaelis–Menten analysis

The mechanism for an enzyme-catalyzed reaction can be summarized in the form [32]:

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \mathrm{ES} \overset{k_2}{\rightarrow} \mathrm{E} + \mathrm{P} \quad (1)$$

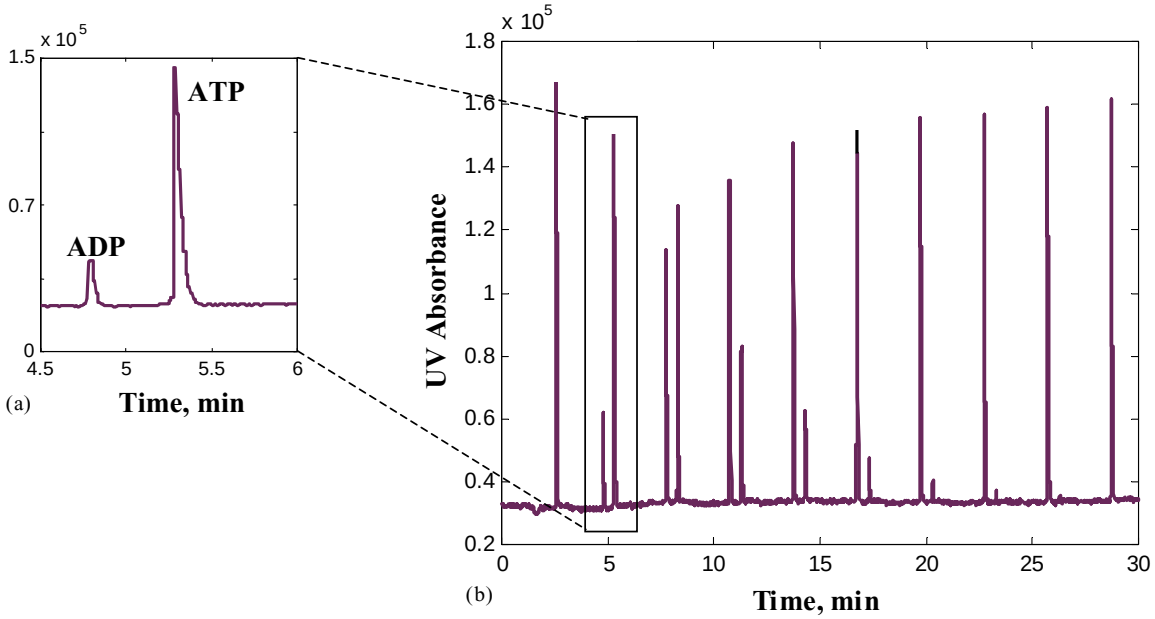

Fig. 4. (a and b) Electropherogram of reaction mixture recorded during the monitoring. Experimental conditions: phosphate buffer 25 mM, 25 mM CTAB, pH 4.5, fused silica capillary: effective length 25 cm × 50 μm i.d., UV detection at 254 nm, potential −25 kV. Sample medium: $NaH_2PO_4 \cdot 2H_2O$ 12 mM, $NaHCO_3$ 4.2 mM, D-glucose 22 mM, $MgSO_4$ 2.7 mM, hexokinase 0.037 U/ml, ATP 100 μM, sample pH = 7.

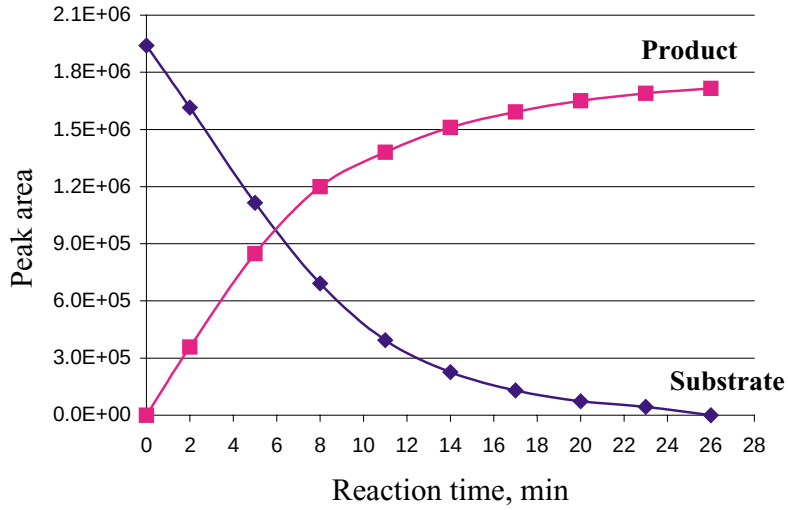

Fig. 5. Reaction progression curves for hexokinase. Curves are based on peak areas and illustrate the increase of product (ADP), and disappearance of substrate (ATP).

In this equation, k_1 is the rate constant for the formation of the enzyme–substrate complex ES, from the enzyme E and the substrate S; k_{-1} the rate constant for the reverse reaction, and k_2 the rate constant of the conversion of the ES complex to product P. The kinetics of the enzymatic reaction was analyzed according to the Michaelis–Menten model, which describes the relation between the initial reaction velocity V and the substrate concentration [S]:

$$V = \frac{[S]V_{max}}{[S] + K_m} \quad (2)$$

where V_{max} is the maximum reaction velocity and K_m the Michaelis constant, the substrate concentration at half the maximum velocity. It follows from Eq. (1) that the Michaelis constant is:

$$K_m = \frac{k_{-1} + k_2}{k_1} \quad (3)$$

The Michaelis constant is a unique parameter of a specific enzyme. It reflects the binding affinity of the enzyme for a specific substrate. The determination of K_m requires the measurement of the initial velocity at several initial substrate concentrations. For this, the reaction mixture, containing 0.04 U/ml of HK, was used and the concentration of ATP was varied between 0.05 and 0.25 mM. Typical reaction progression curves at different ATP concentrations, monitored by measuring the appearance of the product ADP, are illustrated in Fig. 6, where the product peak areas are plotted versus time. The initial reaction velocities were deduced from the slopes of the linear part (first 2–6 min) of these curves. To estimate the precision of the initial velocity determination, the initial reaction rate was determined five times for the 0.1 mM concentration of ATP. R.S.D. value of 5% was obtained, which indicated a good repeatability of this CE method.By linearization of the Michaelis–Menten equation, the Lineweaver–Burk plot is obtained, which describes a linear relation between $1/V$ and $1/[S]$:

$$\frac{1}{V} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \quad (4)$$

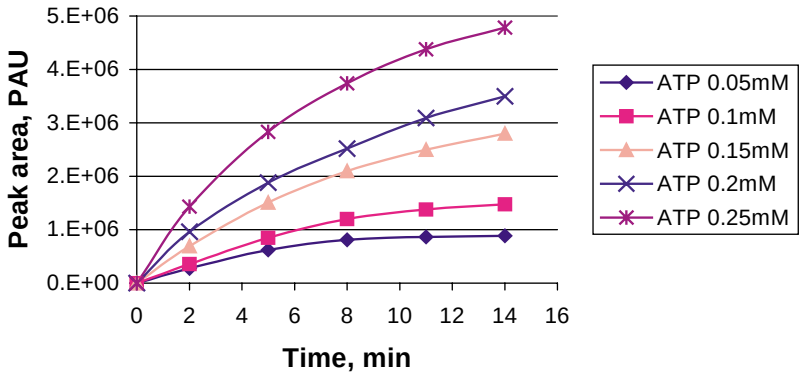

Fig. 6. Formation of ADP during the hydrolysis of ATP. For capillary electrophoresis conditions see Fig. 4.

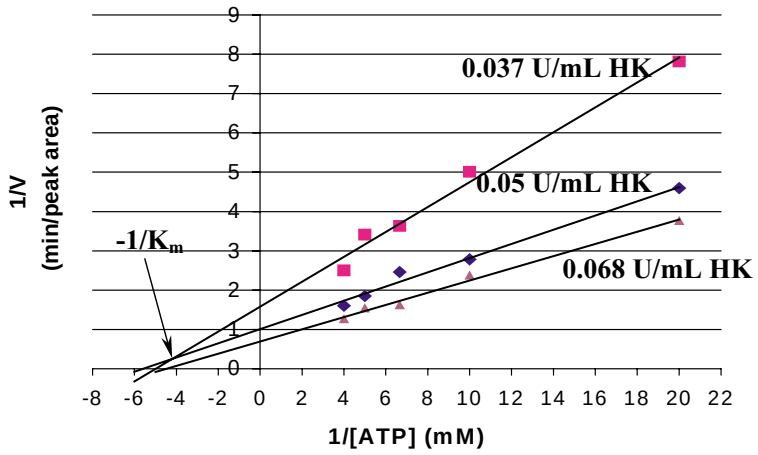

Fig. 7. Lineweaver–Burk plots, for three different HK concentrations (0.037, 0.05 and 0.068 U/ml); The concentration of ATP varied between 0.05 and 0.25 mM. Each concentration was analyzed in triplicate.

The Michaelis constant K_m for ATP hydrolysis was estimated by linear least squares regression from Lineweaver–Burk plots. The described procedure for K_m determinations was repeated for two more HK concentrations (Fig. 7). The K_m values determined for three different enzyme concentrations (0.037, 0.050 and 0.068 U/ml) were 0.20, 0.18 and 0.22 mM, representing an average K_m 0.201 ± 0.015 mM (i.e. $n = 3$, R.S.D. = 7.4%) The correlation coefficients (r) were 0.993, 0.993 and 0.995, respectively. The experimentally obtained K_m value is within the previously reported data, measured in different laboratories by different methods from $K_m = 0.2$ mM [33,34] to 0.4 mM [35,36].

4. Conclusion

A simple and fast CE method for on-line monitoring enzymatic reactions was developed and tested on the conversion of ATP to ADP by hexokinase using micellar electrokinetic chromatography with reversed electroosmotic flow. The analysis of hexokinase activity allowed determination of the enzyme kinetic parameters. This was demonstrated for K_m, which value appeared to be consistent with the previously reported data. The results of this study confirm that CE can successfully be used for studying the enzymatic reaction kinetics. It is rapid and convenient and provides reliable data.

The use of this specially designed CE sampler provides an opportunity for on-line monitoring of the enzymatic conversion without any reaction medium perturbation and sample preparation, thus avoiding the potential errors, resulting from the failure to stop the reaction prior to sample injection, faced in some other assay methods. Moreover, an important advantage of the developed CE method is that the autosampler construction allows the operation with small amounts of the sample for each analysis (microliter range) so, that the amount of sample injected for each analysis does not change the reaction volume significantly. Also the sample can be introduced into column without HV interruptions, a factor essential for good CE reproducibility.

The CE separation of substrates and products seems to be especially advantageous, compared with methods, which use single component monitoring. Where better monitoring and understanding of enzymatic processes is crucial, it provides details about the consumption of substrates and the formation of products simultaneously in a single run. The separation time by using CE is usually shorter and the efficiency is higher than in HPLC, which may also be essential in some applications. Thus, the separation of the nucleotides in this work was completed within 3 min, but can be reduced even further, if necessary. The measuring equipment was stable during a long run, and the reproducibility of the results was acceptable for monitoring purposes.

Clearly, the reaction studied here, ATP conversion to ADP by hexokinase is a well-known textbook example reaction and as such is not of much interest. It has served here as a good reference point for the estimation of capabilities of on-line monitoring equipment, measuring the characteristics of the sampling system and estimation of the overall potential of CE in such experiments. The investigation, carried out, demonstrated that CE indeed could and should be developed into a routine method for an on-line monitoring and control of complex enzymatic processes. Currently, we are interested in application of the on-line pneumatic sampling system for measurements of the kinetics of regulation of mitochrondrial respiration in the muscle cells. The kinetics have been estimated by HPLC off-line [37], but the measurements have been hampered by lengthy sample preparation procedure, which includes precipitation of proteins with trifluoroacetic acid followed by sample neutralization. Also, the difference of more than two orders of reactant and product peak areas and their overlapping limited the precision of quantification of this kinetics. Such peak overlapping would not be the case in CE due to the much higher efficiency of the latter method.

Acknowledgements

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