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Effect of pH on the Stability of Hexokinase and Glucose 6-Phosphate Dehydrogenase

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Abstract

Hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PDH) are important enzymes used in biochemical studies and in analytical methods. The stability of the enzymes can be affected by several variables, pH being one of them. The effect of pH on the stability of HK and G6PDH was evaluated in this work. Baker's yeast cells were suspended in 50 m*M* Tris-HCl buffer (pH 7.5) containing 5.0 m*M* MgCl2 , and submitted to disruption by agitation with glass beads and in the presence of protease inhibitors. The cell-free extract was obtained by centrifugation (2880*g*; 10 min), followed by dilution into the buffers: 0.1 *M* acetate–acetic acid (pH: 4.0, 4.5, 5.0, or 5.5), 0.1 *M* phosphate buffer (pH: 6.0, 6.5, or 7.0), and 0.1 *M* Tris-HCl buffer (pH: 7.5, 8.0, 8.5, 9.0 or 9.5). The residual activity of HK and G6PDH, expressed as μmol of NADPH formed per min, were measured through a period of buffer–enzyme contact from 0 to 51 h at 4°C. It was observed that up to 4 h both enzymes were stable in all buffers used. However, after 51 h HK was stable at pH 6.0 and 7.5, whereas G6PDH was stable at pH 7.0 , 9.5, and between 4.5 and 5.5.

Index Entries: pH; hexokinase; glucose 6-phosphate dehydrogenase.

Introduction

Hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PDH) are enzymes largely found in living cells. Both enzymes are used as analytical tool for measuring glucose, fructose, mannose, ATP, and creatin-kinase activity *(1–3)*. They are also used in wine and fruit juice industries for detecting illegal addition of sugars in the final products *(4)*.

In the case of intracellular enzymes, such as HK and G6PDH, the cell disruption is an obligatory step in their production. But to accomplish that,

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the cells are disrupted in a buffered medium supplemented with protease inhibitors, in order to minimize the losses of enzyme activity. According to Asenjo *(5)* the pH can alter the charge of chemical groups located in the side chain of protein amino acids leading to a net charge modification of the overall macromolecule, changing its solubility properties, at the end. Moreover, the enzyme can lose the catalytic capability either reversibly (precipitation at isoelectric pH) or irreversibly (denaturation at pH values out of the stability pH interval) *(6)*. As during downstream procedures each step takes some time to be carried out , or the cell free extract must be left to stand for a period of time before processing, then the evaluation of the enzyme stability becomes indispensable.

This work deals with the effect of pH (4.0 to 9.5) on the stability of HK and G6PDH present in the cell-free extract left at 4°C for 51 h.

Materials and Methods

Chemicals

Glucose 6-phosphate dehydrogenase, ATP, nicotinamide adenine dinucleotide phosphate ($NADP^+$), phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol, glucose 6-phosphate (G6P) and glucose were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were of analytical grade. The glass beads were purchased from Corning Glass (New York, NY).

Cell Disruption

Cells of commercial pressed ferment (*Saccharomyces cerevisiae*, baker's yeast) were previously rinsed by suspending 150 g of wet cake per liter of 0.145 *M* NaCl at 4°C, followed by centrifugation at 8000*g* for 25 min. Then, the cell cake was suspended in 50 mL of 50 m*M* Tris-HCl buffer (pH 7.5) containing 2.0 m*M* ε-aminocaproic acid, 0.2 m*M* EDTA, 10 m*M* β-mercaptoethanol, 1.0 m*M* phenylmethylsulfonyl fluoride (PMSF), and 5 m*M* MgCl2. A volume of 5 mL of cell suspension was submitted to a vortex (Phoenix AT 56) in the presence of 5 mL of glass beads (diameter = 0.5 mm) and the mixture maintained below 10°C all the time. Cell debris and glass beads were removed by centrifugation (2880*g*; 10 min) and the supernatant was collected.

Hexokinase Activity Assay

In a 1 mL quartz cell were introduced 800 µL of Tris-HCl buffer (100 m*M*; pH = 7.5), 100 µL of glucose (10 m*M*), 40 µL of ATP (5 m*M*), 10 µL of magnesium chloride (10 m*M*), 30 µL of the supernatant, and 10 µL of G6PDH (one Sigma unit). After introducing 10 µL of NADP (1m*M*), the hexokinase activity was followed by measuring the continuous reduction of NADP at 30°C in a spectrophotometer (λ= 340 nm) *(3)*. As the samples were crude extracts, a blank was also made to discount any residual HK activity due to the presence of endogenous substrates

(glucose and ATP), G6PDH, and/or cofactors (Mg^{2+} and NADP). One hexokinase unit was defined as the amount of enzyme catalyzing the reduction of 1 µmol of NADP per min under the assay conditions. The enzyme activity was expressed as U/mL. Each determination was made in triplicate and the standard deviation was 2%. No significant decrease in hexokinase activity was detected after storing the supernatant for 5 h on ice.

Glucose 6-phosphate Dehydrogenase Activity Assay

In a 1mL quartz cell were introduced 850 µL of Tris-HCl buffer (100 m*M*; pH = 7.5), 100 µL of glucose 6-phosphate (10 m*M*), 10 µL of magnesium chloride (10 m*M*), 30 µL of the supernatant. After introducing 10 µL of NADP (1m*M*), the G6PDH activity was followed by measuring the continuous reduction of NADP at 30°C in a spectrophotometer (λ= 340 nm) *(3)*. As the samples were crude extracts, a blank was also made to discount any residual G6PDH activity due to the presence of endogenous substrate (G6P) and/or cofactors (Mg^{2+} and NADP). One G6PDH unit was defined as the amount of enzyme catalyzing the reduction of 1 µmol of NADP per min under the assay conditions. The enzyme activity was expressed as U/mL. Each determination was made in triplicate and the standard deviation was 2%. No significant decrease in G6PDH activity was detected after storing the supernatant for 5 h on ice.

Preparation of the Storage Solution

The supernatant and an appropriate 0.1 *M* buffer solution (acetic acid–acetate buffer pH 4.0, 4.5, 5.0, or 5.5; phosphate buffer pH 6.0, 6.5, or 7.0, and Tris-HCl pH 7.5, 8.0, 8.5, 9.0, or 9.5) were mixed in a volumetric proportion of 1:9. In all tests the pH was checked after homogenizing the mixture.

Samples of the storage solutions were taken for residual enzyme activity determination after 0, 4 h, 8 h, 12 h, 24 h and 51 h of cell-free-extract–buffer contact. It was also calculated the enzyme denaturation coefficient *(k)* by plotting the logarithm of the enzyme activity against the storage time.

Results and Discussion

The data related to the residual activity of hexokinase and glucose 6-phosphate dehydrogenase after leaving the cell-free extract in buffer solutions at different pH by 51 h are presented in Table 1. Taking into account the value of the ratio U_{th}/U_{0h} [where U_{th} and U_{0h} are the HK or G6DPH activity after a certain time (t *h*) and 0 h of storage, respectively] not lower than 90% as an acceptable indicator for the enzyme activity retention against pH *(7)*, we can conclude that both enzymes present in the cell-free extract were stable up to 4 h of storage at pH interval between 4.0 and 9.5 (Table 2). However, after 4 h of storage both enzymes were markedly unstable at pH 4.0, but the opposite at pH 7.0. From Table 2 we can also see that the G6PDH is quite stable at pH interval of 7.0 and 9.5, even after 51 h of buffer–enzyme contact. The hexokinase, in turn, was

Table 1
Residual Activity for Hexokinase and Glucose 6-Phosphate Dehydrogenase Present in the Cell-Free Extract at Different Values of pH

	Time (h)					
pH	0	4	8	12	24	51
4.0	8.89 / 0.54[a]	8.22 / 0.50	7.32 / 0.47	6.59 / 0.44	4.97 / 0.36	0 / 0
4.5	8.39 / 0.52	7.81 / 0.50	7.22 / 0.49	7.11 / 0.46	7.04 / 0.42	6.98 / 0.33
5.0	9.67 / 0.63	9.64 / 0.63	9.58 / 0.61	9.40 / 0.61	9.40 / 0.56	7.83 / 0.55
5.5	10.4 / 0.69	10.3 / 0.69	9.94 / 0.59	9.72 / 0.59	8.40 / 0.59	8.21 / 0.59
6.0	11.8 / 0.79	11.6 / 0.72	11.6 / 0.71	11.5 / 0.71	11.3 / 0.70	11.1 / 0.65
6.5	11.0 / 0.71	10.4 / 0.67	10.2 / 0.67	10.1 / 0.65	10.0 / 0.62	9.40 / 0.61
7.0	11.1 / 0.66	10.8 / 0.66	10.4 / 0.66	10.4 / 0.65	10.3 / 0.64	9.90 / 0.61
7.5	11.0 / 0.71	10.2 / 0.69	10.2 / 0.68	9.42 / 0.67	8.64 / 0.66	8.50 / 0.64
8.0	10.5 / 0.76	10.1 / 0.72	9.77 / 0.72	9.51 / 0.72	8.83 / 0.72	7.36 / 0.65
8.5	10.5 / 0.66	10.0 / 0.66	9.70 / 0.66	9.51 / 0.66	8.50 / 0.66	6.65 / 0.65
9.0	10.2 / 0.70	9.57 / 0.66	9.44 / 0.66	8.97 / 0.65	7.94 / 0.64	5.90 / 0.64
9.5	10.3 / 0.71	9.51 / 0.67	9.12 / 0.66	8.51 / 0.64	7.21 / 0.63	4.87 / 0.63

[a]Hexokinase activity (first value) and glucose 6-phosphate dehydrogenase activity (second value) both expressed as U/mL.

Table 2
The Enzyme Activity Retention Ratio (U_{th} / U_{0h}) for Hexokinase and Glucose 6-Phosphate Dehydrogenase at Different pH

pH	U_{4h}/U_{0h}	U_{8h}/U_{0h}	U_{12h}/U_{0h}	U_{24h}/U_{0h}	U_{51h}/U_{0h}
4.0	92.5 / 92.6*	82.3 / 87.0	74.1 / 81.5	56.0 / 66.7	0 / 0
4.5	93.1 / 96.2	86.1 / 94.2	85.0 / 88.5	84.0 / 80.8	83.2 / 63.5
5.0	99.7 / 100	99.1 / 96.8	97.2 / 96.8	97.2 / 89.0	81.0 / 87.3
5.5	99.0 / 100	95.6 / 85.5	93.5 / 85.5	80.8 / 85.5	79.0 / 85.5
6.0	98.3 / 91.1	98.3 / 90.0	97.5 / 90.0	95.8 / 88.6	94.1 / 82.3
6.5	94.5 / 94.4	92.7 / 94.4	91.8 / 91.5	91.0 / 87.3	85.5 / 86.0
7.0	97.3 / 100	93.7 / 100	93.7 / 98.5	92.8 / 97.0	89.2 / 92.4
7.5	92.7 / 97.2	92.7 / 95.8	85.6 / 94.4	78.5 / 93.0	77.3 / 90.1
8.0	96.2 / 94.7	93.0 / 94.7	90.6 / 94.7	84.1 / 94.7	70.1 / 85.5
8.5	95.2 / 100	92.4 / 100	90.6 / 100	81.0 / 100	63.3 / 98.5
9.0	93.8 / 94.3	92.5 / 94.3	87.9 / 93.0	77.8 / 91.4	57.8 / 91.4
9.5	92.3 / 94.4	88.5 / 93.0	82.6 / 90.1	70.0 / 88.7	47.3 / 88.7

[a]Activity retention ratios, expressed as percentage, for hexokinase (first value) and glucose 6-phosphate dehydrogenase (second value) .

Table 3
Values of the pH Inactivation Constant (k_{pH})
for Hexokinase (HK) and Glucose 6-Phosphate Dehydrogenase (G6PDH)

pH	HK	G6PDH
4.0	-107×10^{-4}[a]	-73×10^{-4}
4.5	—	-38×10^{-4}
8.0	-29×10^{-4}	—
8.5	-38×10^{-4}	—
9.0	-46×10^{-4}	—
9.5	-63×10^{-4}	—

[a] k_{pH} expressed as h^{-1}.

sensitive to pH values higher than 7.5. These results are quite similar to those found by Silva et al. *(8)* for the purified forms of HK (SIGMA®-H5375) and G6PDH (SIGMA®-G8529). Probably, the macromolecular structures of these proteins are well adapted to less acidic pH values, because the pH of the yeast cell cytoplasm, their natural location, is normally buffered at pH of 6.5–7.5 *(9)*.

Another point to be borne out is that the best stability pH values are higher than the correspondent isoelectric points of the enzymes, which are 4.8 and 6.0, respectively, for HK and G6PDH *(10–11)*. Then, for pH ≥ 6.5 we can assume that the effective electrical charge of both proteins is negative, a favorable condition for planing an extraction protocol on the extraction of HK and G6PDH from yeast cell-free extract through an aqueous two-phase system (for instance, the aqueous PEG-phosphate mixture) (12).

By plotting the logarithm of activity (log v) against time it was possible to determine the pH denaturation constant (kpH) for both enzymes, whose values are presented in Table 3. As log v= f(t) is linear (Figs. 1 and 2) , so kpH is a typical first order constant, at least at the values of pH indicated *(13)*. Therefore, it can be predicted with some accuracy the loss of HK and G6PDH activity after a certain period of time (from 0 to 51 h, at least) of buffer–enzyme contact. This information is important because enable to set the adequate manipulation time for the extraction of both enzymes through the aqueous two-phase system.

Conclusion

From the data presented two main conclusions could be enhanced. First, both enzymes must be stored in buffer solution at neutral pH. Second, as k_{pH} is a first order constant the aqueous two-phase extraction procedure can properly be planned so that the loss on enzyme activity is minimized.

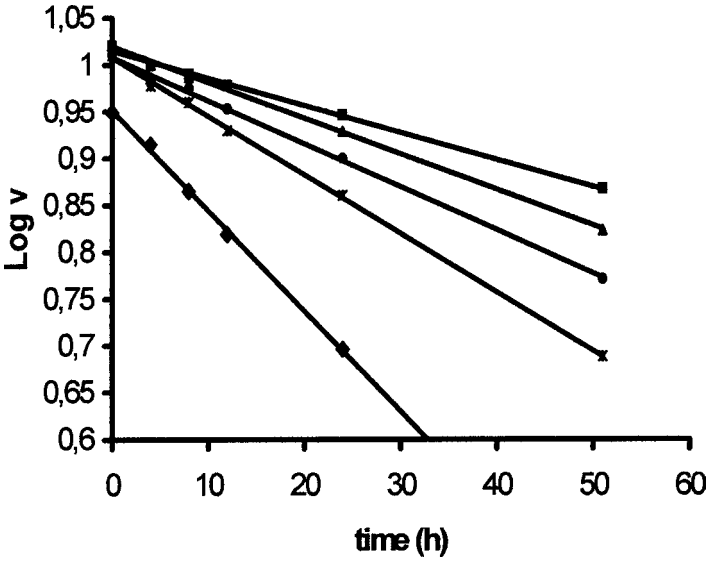

Fig 1. Evaluation of the pH denaturation constant related to HK through the log v × time plot for different values of pH: 4.0 (◆), 8.0 (■), 8.5 (▲), 9.0 (●) and 9.5 (✱).

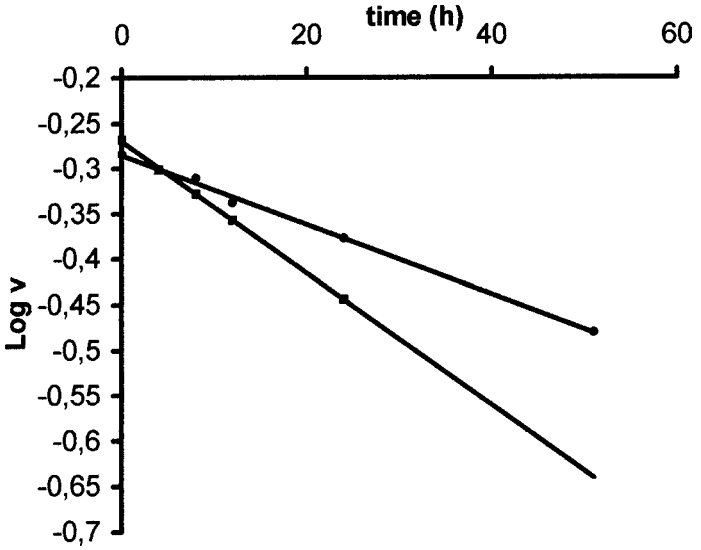

Fig. 2. Evaluation of the pH denaturation constant related to G6PDH through the log v × time plot for different values of pH: 4.0 (■) and 4.5 (●).

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