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Probing Conformational Feature of a Recombinant Pyruvate Kinase by Limited Proteolysis

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Abstract

Pyruvate kinase is a key enzyme in glycolytic pathway that catalyzes the transphosphorylation between phosphoenolpyruvate and ADP to yield ATP and Pyruvate. Geobacillus stearothermophillus has a stable pyruvate kinase with determined crystal structure that composed of four separate domains. Given that limited proteolysis experiments can be successfully used to probe conformational features of proteins, in this study we obtained useful information on Geobacillus pyruvate kinase using limited proteolysis with two proteases that have different substrate specificity and optimum temperature of activity, trypsin and thermolysin. Proteolytic patterns at different temperatures indicate that resulting fragments were the same but the rate of digestion increased with temperature. In the next step, Sucrose and Glycine were used to examine the effects of additives on stability and activity of pyruvate kinase. Limited proteolysis was carried out at 37 °C by trypsin and at 30, 55 and 60 °C in presence of thermolysin, in the absence and presence of different concentrations of sucrose (0– 1.5 M) and glycine (0–1.5 M). We observed that stabilization of pyruvate kinase by this osmolytes is concentration dependent and the rate of limited proteolysis in presence of additives, at temperatures above 60 °C decrease; however, there was no any effect on proteolytic patterns. In all experiments the activity of pyruvate kinase was determined with a couple assay methods by luciferase. A clear correlation was observed between proteolytic digestion and enzyme activity. This study reveals a number of flexible and protease-prone regions of pyruvate kinase that exist regardless of the environmental conditions.

Keywords: Pyruvate kinase; Limited proteolysis; Additives; Bioluminescence

Introduction

Pyruvate kinase (PK, EC 2.7.1.40) catalyzes the final step of glycolysis that is essentially irreversible: transphosphorylation from phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP [21, 45]:

$$\text{PEP} + \text{Mg-ADP} + \text{H}^+ \xrightarrow{\text{PK, K}^+} \text{Mg-ATP} + \text{Pyruvate}$$

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This enzyme plays an important role in the control of the metabolic flux from fructose-1, 6- bisphosphate (Fru-1, 6- P_2) to pyruvate and may be considered as a key enzyme not only for the glycolytic pathway but also for the entire cellular metabolism [28].

Better understanding of structural features and specificity of Pyruvate kinase will help to take advantage of the enzyme. PK have been isolated and characterized from a number of prokaryotes and eukaryotes, mostly in form of tetramers of identical subunits [17, 21, and 40]. There are a few bacterial PK with determined 3D structure. This enzyme is relatively unstable and *Geobacillus stearothermophillus* PK (GstPK) is the most stable bacterial PK with determined crystal structure. Each subunit of GstPK is composed of four separate domains. The first domain, A, is located between the B and C domains that was formed by two separate stretches of amino acids that fold together into a common parallel $(\beta/\alpha)_8$ barrel motif with two additional α-helices, and is supposed to be the catalytic domain. The second domain, B, is a capped domain, comprised of a small four-stranded β-barrel motif that forms a cap over the active site. The binding site for an allosteric effector is completely located in the C domain that is comprised of an α/β open-sheet motif. Finally, the extra C terminal sequence formes a new domain that is located at the C terminus of the protein and is named the C' domain [41].

It has been demonstrated that PK can serve as a biosensor [11] or as a target for chemotherapy [17], improving folic acid and amino acids production [39, 46]. PK is also used in continuous-flow ATP amplification system to increase the sensitivity of bioluminescence assay [38]. Furthermore, PK can be used in a coupled bioluminescence assay with firefly luciferase for ADP measurement [30].

We have recently evaluated limited proteolysis of firefly luciferase, as the first enzyme in the coupled bioluminescent assay for ADP measurement, in the absence and presence of additives [4,5]. Moreover, after site-directed mutagenesis, its becomes more stable against trypsin proteolysis [35].

In the current investigation study, limited proteolysis experiments are used to examine the flexible and exposed regions of a thermostable PK. This is based on the hypothesis that limited proteolysis occurs exclusively at "hinge and fringes" [19] and conformational parameters such as accessibility and segmental mobility correlate quite well with proteolytic sites [14,16,32,42]. Thus Limited proteolysis experiments are used to probe conformational features of this enzyme. The effects of additives including sucrose and glycine on the resistance of PK against proteolysis digestion are also studied.

Materials and Methods

Chemicals

All of the following materials were purchased from Sigma (St. Louis, MO): ADP, $MgSO_4$, $MgCl_2$, PEP, 2-Mercaptoethanol, TEMED, Tris, BSA, IPTG, and other chemicals were obtained from Merck (Darmstadt, Germany). D-luciferin was purchased from Synchem and trypsin was obtained by Fluka.

Expression of Recombinant Pyruvate Kinase

In order to purify pyruvate kinase, *E. coli* BL21 cells harboring recombinant plasmid were grown at 37 °C in 10 mL of terrific broth (TB) medium, containing 50 μg mL^{-1} of kanamycin. After overnight culture, 2 ml of the medium was transferred to 200 mL of fresh TB medium. The growth continued at 37 °C with vigorous shaking until absorbance at 600 nm (OD_{600}) reached 0.9, the culture was then induced with lactose (4 mM) and IPTG (to a final concentration of 1 mM), and the incubation was continued for an additional 12 h at 37 °C. Cells were harvested by centrifugation at 2500 g for 30 min at 4 °C, suspended in five volumes of lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, pH 7.8) containing 1 mM phenylmethyl-sulfonyl fluoride (PMSF).

Pyruvate Kinase Purification Using Affinity Chromatography

The cells were disrupted with a Misonix sonicator, debris were removed by centrifugation at 8000 g for 20 min at 4 °C. The supernatant was applied to nickel–nitrilotriacetic acid (Ni-NTA) Sepharose column as described by the manufacturer (Qiagen, Inc). The purified enzyme had purities more than 95% and was stored in 10% glycerol at -20 °C for further use.

Determination of Protein Purity and Concentration

The purity of the fractions containing purified pyruvate kinase was determined by SDS-PAGE by using 12% SDS-polyacrylamide gel electrophoresis according to Laemmli [24]. Proteins were visualized by coomassie blue staining. The concentration of purified protein was measured by the method of Bradford with bovine serum albumin as the standard [7].

Tryptic Hydrolysis

Digestion of pyruvate kinase with trypsin was

carried out in 25 mM Tris/HCI pH 7.8 at 37°C in different time intervals, The ratio of protein to protease was 30:1.

Thermolytic Hydrolysis

Digestion with thermolysin was carried out in the same buffer as tryptic digestion and in different time intervals and at various temperatures ranging over 30 to 65 °C. The ratio of protein to protease was 150:1.

In both digestion experiment, at the end of incubation time, aliquots were removed from the reaction mixture and were immediately denatured by boiling in SDS-β-mercaptoethanol, and samples were then ran on SDS-PAGE. The gels were stained with coomassie brilliant blue R-250.

Remaining Activity of Pyruvate Kinase Throughout Limited Proteolysis

Remaining enzymatic activity was measured in aliquots taken at suitable time intervals in a luciferase coupled assay system by measuring the light emission. Samples were incubated with trypsin ([E / S] ratio 1:30) and or thermolysin ([E / S] ratio 1:150) at 25 mM Tris buffer, pH 7.8 containing 10 mM $CaCl_2$, at different temperatures (37 °C for tryptic and 30, 55, 60 and 65 °C for thermolytic digestion), or in the presence and absence of glycine and/or sucrose (1/5 M) as additives. At regular intervals, samples were removed, and the remaining activity was measured, immediately. Assays were initiated by injecting samples in enzyme assay buffer (10 mM Tris-HCl, 7 mM ADP, 7 mM PEP, 10 mM $MgCl_2$, 10 mM KCl pH 7.8) at 25 °C for 5 min. Then 2 µL of the diluted sample was added to 38 µL of the substrate solution (10 mM Tris-HCl, 2 mM D-luciferin, 10 mM $MgSO_4$, 10 mM KCl and pH 7.8). The ATP produced by PK reaction was measured by adding luciferin- luciferase using a Sirius Single Tube Luminometer (Berthold Detection systems, Germany), as reported earlier [31-2] of the enzyme in the absence of proteases was considered as control (100%).

Thermolysin Activity Measurements

The activity of thermolysin in the absence and presence of osmolytes was determined at 60 °C, in 50 mM Tris buffer, pH 7.8, in 0.5 ml reaction volume, based on Matsubara method [27]. The reaction was carried out with 0.5% casein as substrate and 100 µg/ml thermolysin concentrations. After 15 and 30 min, the enzymatic activity of thermolysin was halted with 0.5 ml of 10% trichloroacetic acid (TCA) and subsequently, absorbance of the supernatant was measured at 280 nm.

Results

Expression and Purification

Over expression of PK was carried out in the BL 21 host. The purification of the His tag fusion PK was performed by affinity (Ni-NTA-Sepharose) chromatography. The purified PK had purity more than 95% based on analyses by SDS-PAGE in which PK was present as a single band of about 62 kDa (data not shown).

Proteolytic Digestion of Pyruvate Kinase from a Thermostable Geobacillus

In this study, trypsin and thermolysin were used to identify the flexible and exposed region of pyruvate kinase from a thermophilic *Geobacillus*. The intact purified proteins were incubated individually with trypsin and thermolysin under various experimental conditions of protease to pyruvate kinase ratio, temperature and duration of incubation, and the extent of the enzymatic digestion was estimated by SDS-PAGE.

Tryptic Digestion

When PK was incubated at 37 °C in the presence of trypsin, enzymatic activity of PK decreased up to 50% after 60 min with appearance of smaller fragments in SDS-PAGE (Fig. 1). In general, these fragments are relatively stable against tryptic digestion and the effect of limited proteolysis with trypsin is not complete and even after about 360 min a high amount of intact enzyme remained.

It was realized that conformational change of PK caused by thermal treatment (at 60 °C for 15 min, data not shown) is irreversible. In the next stage, the effect of thermal treatment on proteolytic pattern was examined. However, no differences in proteolytic pattern were seen (Fig. 2).

Thermolytic Digestion

Since Geobacillus PK is a thermophilic enzyme, with an optimum temperature of 65 °C, it was thought it might be interesting to carry out limited proteolysis experiments at temperatures higher than 37 °C. The protease chosen was thermolysin since not only it is active within a broad range of temperatures [27] but also it has broad specificity for substrate and thus it is a more reliable protease for probing conformational properties. Thermolytic digestion was performed at

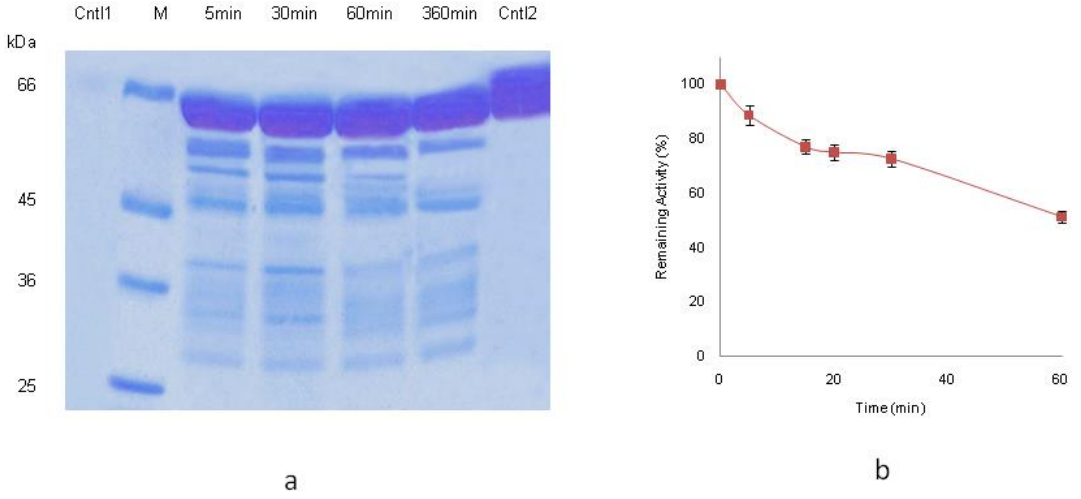

Figure 1. Limited proteolysis in presence of trypsin. a: SDS-PAGE of the tryptic digestion of PK. The molecular weights of the fragments corresponding to digestion of enzyme and marker (M) are indicated. Cntl1: trypsin in a concentration that was used in experiment. Cntl2: PK without trypsin. b: Loss of PK activity during incubation with trypsin. The error bars show the variation between various reactions.

different temperatures according to aggregation studies that indicate that temperatures ranging from 30 to 65 °C are reliable for proteolytic experiments (data not shown).

As shown in Fig. 3a, the rate of thermolytic digestion at 60 and 65 °C is very high. In 60 °C after 5 min, 3 distinct fragments of about 36, 50 and 56 kDa are detected. Over a period of time the 56 kDa fragment disappeared very soon, and was not seen even at 65 °C. The concentration of the other two fragments increased, the 50 kDa fragment was susceptible to more thermolytic digestion and completely disappeared after 30 min but 36 kDa fragments was stable. The reduction in activity is compatible with digestion results (Fig. 3b) and reaches about 0% after 20 minutes. Thus the 36 kDa fragment has not any activity alone.

These experiments were continued with thermolytic digestion at 55 °C (Fig. 4). Like tryptic digestion, fragments appeared immediately after incubation, but in the first 30 min of reaction, no significant reduction in residual activity was seen. Because PK is a thermophillic enzyme, activation at 55 °C is more than inactivation at first 30 min of thermolytic digestion.

As shown in Fig. 5, similar to higher temperatures, the concentration of 56 kD fragment is decreased (in a lower rate) but the concentration of 50 and 36 kD fragments increased respectively and 36 kD fragment is fully resistant to proteolysis and has no more susceptible region for thermolytic digestion.

Further experiments were done in order to examine the effects of temperature in proteolysis. Thus, limited proteolysis in presence of thermolysin was performed at two different temperatures, 55 and 30 °C, and compared with each other (Fig. 6). At low temperatures, the rate of digestion is slower and high amounts of intact enzyme remained after the experiment.

Comparison of Limited Proteolysis in Presence of Trypsin and Thermolysin

One of the most important points in this study is comparison of PK to different proteases. Comparison of

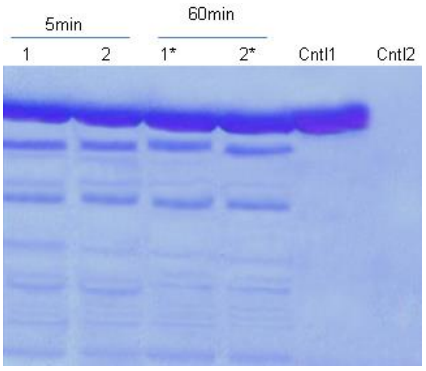

Figure 2. Effect of thermal treatment on tryptic digestion. 1 and 1*: without thermal treatment. 2 and 2*: with thermal treatment. Cntl1: PK in absence of trypsin. Cntl2: trypsin in concentration that was used in experiment.

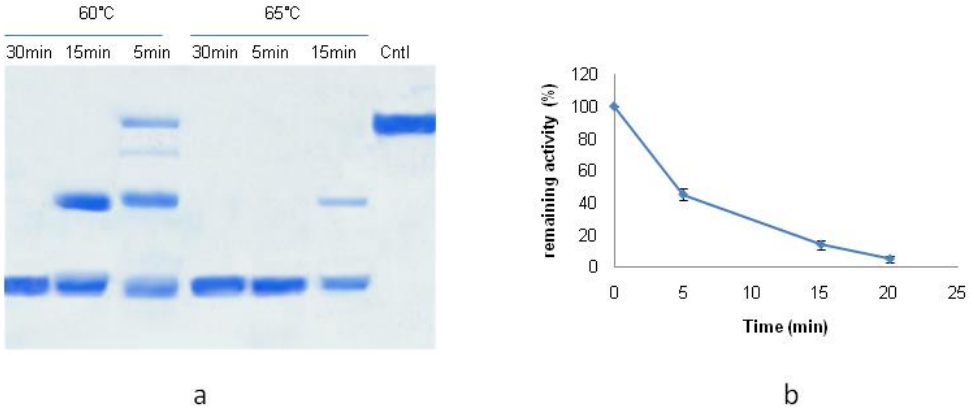

Figure 3. Limited proteolysis in presence of thermolysin at 60 °C and 65 °C. a: SDS-PAGE of the thermolytic digestion of PK. Proteolysis was conducted at 60 °C and 65 °C (protease to substrate ratio [E/S]; 1:150 by concentration). Cntl1: PK without trypsin. b: Loss of PK activity during incubation with thermolysin. The error bars show the variation between various reactions.

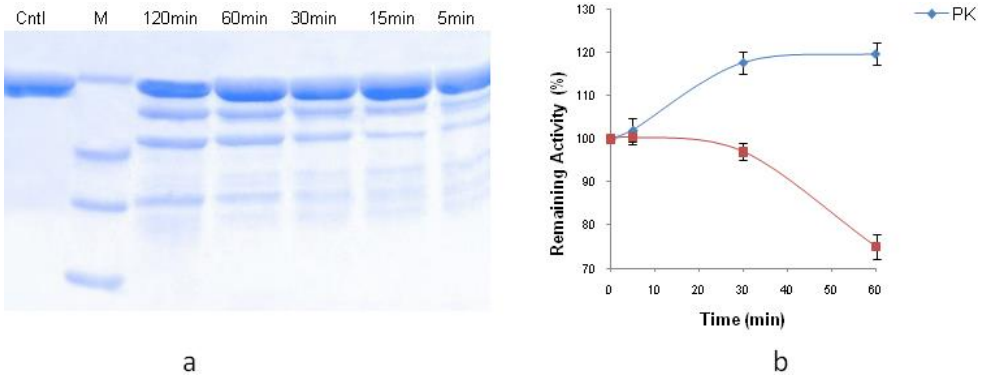

Figure 4. Limited proteolysis in presence of thermolysin at 55 °C. a: SDS-PAGE of the thermolytic digestion of PK that was performed at 55 °C. M: Molecular weight marker, Cntl1: PK without thermolysin. b: residual activity of PK in presence (red line) or absence of thermolysin (blue line) at 55 °C. The error bars show the variation between various reactions.

tryptic and thermolytic digestion reveals the grouping of the preferential cleavage sites within almost the same region of the protein regardless of the kind of proteases used (Fig. 7). In either proteolytic digestion, two fragments of about 56 and 50 kDa were seen, but 36 kDa fragments is present only in thermolytic digestion. This shows that this fragment has no tryptic digestion site on PK in experimental condition.

PK Behavior in Presence of Additives

Limited proteolysis was carried out at 37 °C by trypsin and at 30, 55 and 60 °C in presence of thermolysin, in the absence and presence of different concentrations of sucrose (0– 1.5 M) and glycine (0–1.5 M). In temperatures below 60 °C, protection against proteases were not seen (data not shown), but at 60 and 65 °C limited proteolysis was delayed. It has been observed that stabilization of PK by osmolytes is concentration dependent (data not shown). The increase in concentration of osmolytes increases the stability of the PK enzyme. Analysis indicates that 1.5 M sucrose and glycine are the best concentrations (optimum concentrations) to keep PK against proteolysis. Thus further investigations were performed only with these concentrations. A comparative analysis of coomassie-stained SDS-PAGE (Fig. 8a) clearly reveals that rates of proteolysis are different. In the presence of glycine, the PK is somewhat more resistant to proteolysis than sucrose. PK residual activity that was measured over time in presence of optimum concentrations of sucrose and glycine (Fig. 8b) indicates lower inactivation of PK at 60 °C, especially in presence of glycine.

Influence of Additives on Protease Activity

In order to examine the effects of additives on activity of thermolysin, its activity was measured using casein as substrate in the presence of additives. As shown in Fig. 9, both protease activities were reduced in the presence of sucrose and glycine. It is suggested that less proteolytic degradation may derive from protease inhibition in the presence of additives.

Discussion

During recent years, the applications of PK in various biochemical fields are increased parallel with findings about its conformational features. Among the various techniques in current use, the simple biochemical approach relying on the use of proteolytic probes appears to present significant and specific advantages, which derives from low demands for complex or expensive instrumentation, as well as from the requirement of only minute amounts of protein sample. PK from *Geobacillus* is a suitable sample for such studies due to its thermostability and furthermore, it's known amino acid sequence, and available model of tertiary and quaternary structures.

All experiments were performed under a vast array of different conditions because, in some cases, several bonds might be accessible to proteolysis whilst under slightly different experimental conditions, other bonds or just a single site may be observed [1, 6, 15, 23, 29].

Two pathways for degradation of the compact, globular structure of folded proteins and its relation to protein stability have been proposed [25]. Accordingly, in tryptic digestion (Fig. 1), it seems that initial large fragments are relatively stable and unlike our previous study on firefly luciferase are not exposed to subdigestion. Our attempts in tryptic digestion of PK generated minimal peptide cleavage, and even after 6 h no difference in proteolysis pattern was observed for treated protein under thermal conditions (Fig. 2). In fact, thermal unfolding did not lead to exposure of new susceptible regions on PK.

The primary factor affecting the proteolysis of proteins is the mobility of protein structure [14]. High temperatures increase flexibility and, therefore, the proteolytic susceptibility of a protein [33]. On the other hand, proteases such as thermolysin with wide substrate specificity are more suitable for conformational analysis. Proteolytic experiments in presence of thermolysin at higher temperatures (Figs. 3a, 4a and 5) show 3 distinct fragments. Despite of broad substrate specificity of thermolysin, the appearance of smaller fragments indicate cleavage selectively due to specific conformational features that are compatible with other studies [15, 16, 18 and 34]. It may be suggested that structural constraints prevent access to all possible cleavage sites of PK and thereby retain significant structural integrity at higher temperatures.

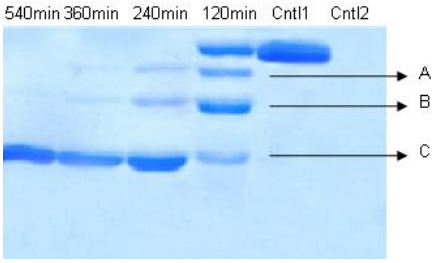

Figure 5. Time course of thermolytic digestion of PK and its fragments at 55 °C.

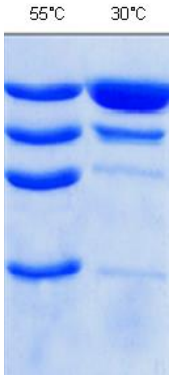

Figure 6. Comparison of Limited proteolysis of PK at 30 and 55 °C.

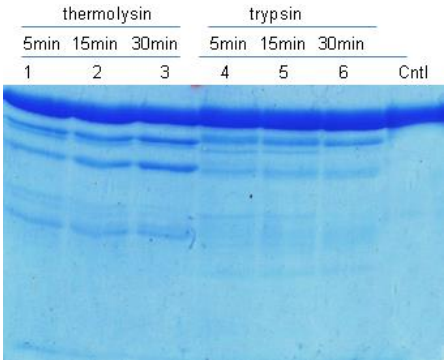

Figure 7. SDS-PAGE of limited proteolysis of PK in presence of thermolysin (lanes 1, 2 and 3, in 55 °C) and or trypsin (lanes 4, 5 and 6, in 37 °C). Cntl: PK in the absence of proteases.

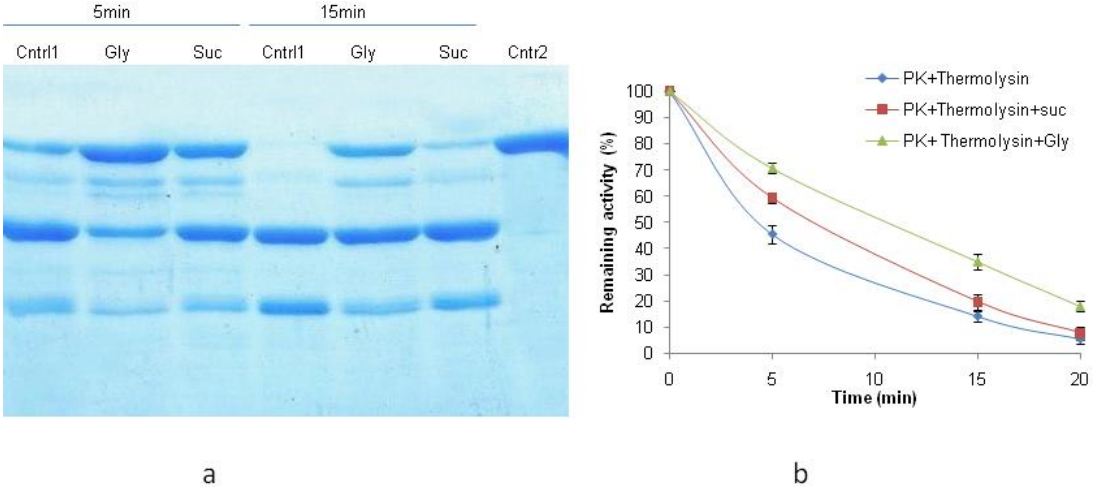

Figure 8. Influence of optimum concentration of osmolytes on the protease susceptibility of PK at 60 and 65 °C. For further detail see material and methods.

In general, building blocks are contiguous sequence fragments of variable sizes, and their stability derives from local interaction. Some building blocks are highly stable; whereas others may be only marginally stable [43]. In the case of thermolytic digestion, the concentration of fragments changes gradually and finally disappears except the smallest fragment (36 kDa), a fragment that was not observed in tryptic digestion but remained resistant to sub digestion with thermolysin, probably due to structural rigidity.

Comparison of proteolytic patterns at different temperatures (Figs. 3a, 4a and 6) indicates that the rate of digestion increased with temperature and PK is a better substrate for thermolysin at high temperatures rather than at low temperatures because of its higher flexibility, but resulting fragments were the same.

According to previous studies [28, 41] PK represents a flexible protein with allosteric transition including large domains and subunit rotations. In previous studies, [10, 36] indicated that substrate binding to PK, causes conformational changes resulting in the exposure or shielding of residues susceptible to modification, but in this study the lack of change in proteolysis pattern shows that the conformation of this thermostable PK at higher temperatures is not subject to inducible conformational changes. In another words, the conformational changes in various temperatures have no significant effect on susceptible regions.

A clear correlation was observed in proteolytic digestion and concomitant activity measurements (Figs. 1b, 3b and 4b). The rate of activity decrease while degradation rates were similar, in a specific period of time, except in thermolytic digestion, at 60°C that shows delayed loss of activity due to its thermostability. Activity of PK is decreased immediately after initiation of proteolytic digestion. It has been shown that activity of *Geobacillus* PK is dependent on simultaneous and concerted rotations of all subunits and domains [26, 28 and 45], so, as a consequence of proteolysis, the correlations between subunits vanished and the enzymatic activity disappeared. In another word, each of fragments could not be active alone and enzymatic activity needs coexistence of all parts of PK.

Since two proteases with different specificities are used in this study, we could create conditions where the

Figure 9. Limited proteolysis in presence and absence of 10mM Mg2+. Thermolytic digestion was performed in presence (lane 1) or absence (lane 2) of 10mM Mg2+ in the same condition, at 55 °C.

sensitivity of the cleavage is not the specificity of the enzyme. Bioinformatics comparison of the putative peptide cleavage sites of PK using peptide cutter software, confirmed more thermolytic cleavage sites (hydrophobic residues) compared to trypsin cleavage sites (data not shown). Proteolytic digestion results (Fig. 7) suggest the preferential cleavage sites within the nearly same region of the protein regardless of the proteases used, that supports the previous studies [3,9,12].

It has been reported that protein resistance to proteolytic attack increases with its conformational rigidity due to the fact that susceptibility to proteolysis reflects the segmental mobility [13, 14, 22 and 42]. In presence of osmolytes (Fig. 8) resistance to proteolysis was increased indicating less proteolytic susceptibility without changes of cleavages sites.

Another part of our data supports the role of Mg^{2+} in conformational changes. Due to susceptibility of PKs in low concentration, in previous studies, advantages were taken from ligands [37, 44]. Thermolytic digestion data in presence of Mg^{2+} (Fig. 9) indicate that, the conformational changes that result from ligand binding are more effective than environmental condition in changing the susceptible region. These results are compatible with several related studies [8, 20], so we suggest that for stabilization of PK, the use of ligands is better than additives that effect environmental condition of PK.

This study summarizes the results that have been obtained from utilizing proteolytic probes of the structure and dynamics of a thermostable PK. Limited proteolysis technique is simple to use, modest in demands for protein sample requirements, instrumentation and experimental effort and, moreover, it provides data on the solution structure of protein, even if the data do not reach the high-resolution level of other physicochemical techniques. The results presented in this manuscript describe new insights into structural properties of thermostable PK in presence of proteases and osmolytes.

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