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Heat shock induction of a 65 kDa ATP-binding proteinase in rat C6 glioma cells

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ABSTRACT

The 45, 55, 65 and 100 kDa ATP-binding proteinases (ATP-BPases) of the heat-shocked (44 ℃ for 30 min, recovery for 12 h) rat C6 glioma cells were purified by DEAE-ionexchange and ATP-affinity chromatography. Their molecular masses, isoelectric points (pI), pH-optima and other properties were analyzed by native proteinase gels. It was shown that the 65 kDa ATP-BPase is specifically induced by heat shock and not detectable in control cells. Its N-terminal 1-9 amino acid sequence was determined by Edman degradation, but no homologies to other proteins in the protein data bases were found. 30 and 31 kDa proteinases can be cleaved from the 45, 55 and 65 kDa proteinases to which they are linked. A possible relationship of the heat-induced 65 kDa ATP-BPase with the ATP-dependent proteinases (ATP-DPases) in prokaryotes and eukaryotes is discussed.

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ABBREVIATION: ATP-dependent proteinases (ATP-DPases) are those proteinases, which can bind to ATP and whose proteolytic activity depends on ATP. The 45, 55, 65 and 100 kDa proteinases of the C6 cells reported in this paper can bind to ATP too, but it was not established that their activity depends on ATP. Thus, it is better designate them as ATP-binding proteinases (ATP-BPases).

Key words: *Rat C6 glioma cells, ATP-binding proteinases, heat shock induction, native proteinase gels.*

INTRODUCTION

Rat C6 glioma cells (C6 cells) are induced by the injection of N-nitrosomethylurea in rats[1]. Although C6 cells are transformed cells, they are good models for many studies on normal animal cells, such as antigen properties, enzymic features, response to hormones, stress, etc. C6 cells share many characteristics with astrocytes and oligodendrocytes[2]. There are many reports on their culture, growth and metabolism[3-5]. However, up to now, we know nothing about their ATP-BPases except calpain[6] and acidic lysosomal proteinases[7].

Proteolysis is essential to life[7],[9] and it is also an essential component in the cellular response to starvation and stress[10]. It is now well established that important pathways of the cellular proteolytic systems are the ubiquitin-dependent selective proteinases in cytoplasmic proteasomes[11] and the selective and non-selective proteinases in lysosomes[12-14]. The ubiquitin-dependent and lysosomal selective proteolytic systems all require ATP to degrade proteins[15],[16]. It has been shown that heat shock can activate the multicatalytic proteinases (proteasomes)[11] and lysosomal enzymes[7],[17] in mammalian cells. And another group of proteinases, named ATP-DPases, have been discovered in recent years. They are closely related to the specific processing and selective degradation of proteins[18] and may play an important role in the cellular stress response[19]. The properties and kinetics of the ATP-BPases in heat shock induction of the C6 cells were analyzed in this paper. These results will afford useful data for further analyzing the action mechanism of HSP68, the lysosomal and ATP-BPases in heat shock response of the C6 cells.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from Sigma (Eastern reagents company, Beijing) unless otherwise noted.

Cell culture and heat shock

Rat C6 glioma cells were grown and recovered after heat shock in DMEM (Sigma), supplemented with 10 % newborn calf serum (NCS, GIBCO), at 37 ℃ in humidified 10 % CO_2 atmosphere.

The cells (10^5/dish) were grown in f 90 mm culture dishes for 3 d. The medium was changed every 3 d. When C6 cells were confluent, the medium was changed again and the C6 cells were immediately treated by heat shock (waterbath of 40, 41, 42, 43, 44, 45 or 46℃ for 15, 30 or 45 min respectively). After recovery at 37℃ for 2, 4, 8, 12, 24, 48 or 72 h respectively, their ATP-BPase activities were determined by the following method.

Native proteinase gels

The heat-shocked C6 cells (44℃ for 30 min, recovery for 12 h) were washed three times by 0. 1 M Tris, pH 7.2, and harvested in the same buffer. Cells were disrupted by sonication (5 W, 15 sec) and the amount of protein in the homogenate determined by the method of Neuhoff et al. [20]. Equal amounts of protein were then separated electrophoretically. Proteinase activities were analyzed in renatured gelatin gels (G-PAGE, 50μg of protein/lane) as described by Heussen and Dowdles[21],[22].

Proteinase pI was detected in two-dimensional renatured gelatin gels (2D-G-PAGE or IEF-G-PAGE, 50 μg of protein/gel) as described by Stein et al.[23], but the buffer used for IEF contained 2.5 M urea and the gel was 1 mm thick.

The optimal condition for determining the activity of ATP-BPases was 24 h incubation at 37 ℃ in a medium containing 0.1 *M* glycine, 5 m*M* $CaCl_2$, 4 mM $MgCl_2$, 1 mM ATP, pH 8.5. This method allowed an estimate of proteinase activities associated with different zones of the renatured gelatin gels.

ATP-BPase purification

The cells were treated with low percolation (20 m*M* Tris-HCl, pH 7.5) for 2 h, then disrupted by sonication (5 W, 15 sec) for 2 min. The homogenate was centrifuged at 20,000 g for 30 min and filtered through a cellulose membrane (4.5 μm pore size). The filtrate was passed through a DEAE-ion-exchange column. The fraction collected after washing with buffer A (80-160 mM NaCl, 10 m*M* $MgCl_2$, 20 m*M* Tris-HCl, pH 7.5) was then applied to an ATP-affinity chromatography column. The fraction collected after washing with buffer B (10 m*M* EDTA, 500 m*M* NaCl, 20 m*M* Tris-HCl, pH 7.5) was then desalted and freeze-dried for use in further experiments.

ATP-BPase activity analysis

Total activity of the ATP-BPases purified from heat-shocked (44℃ for 30 min, recovery for 12 h) C6 cells was analyzed using serum albumin as substrate. The proteinases were incubated in a buffer (0.1 M glycine, 5 m*M* $CaCl_2$, 4 m*M* $MgCl_2$, 1 m*M* ATP, different pH) with serum albumin (0.5 g/L) at 37 ℃ for 6 h. The proteinase activity was calculated by determining the undegraded serum albumin in the buffer[7].

Individual proteinase activities in G-PAGE were determined densitometrically. Gels after G-PAGE were incubated in above buffer at 37 ℃ for 24 h, then the proteolytic zones of the gels were determined by densitometric method and corresponding proteinase activity was calculated. Inhibitors including 10 m*M* EDTA-Na_2, 2 μ*M* a1-antitrypsin (sigma), 2 μ*M* pepstatin and 0.2 m*M* hydroxymercuribenzoate (HMB) were used as described[24],[25] to analyse the characters of those proteinases.

Analysis of the N-terminal 1-9 amino acid sequence of the 65 kDa ATP-BPase

The purified 65 kDa ATP-BPase was separated by two-dimensional SDS-PAGE and then transferred to a PVDF membrane (Bio-Rad, No. 162-0180) using a Trans Blot cell (Bio-Rad). The membrane was stained and destained according to the method of Hobohm et al.[26]. The spot containing the 65 kDa ATP-BPase was cut out and analyzed in a Knauer protein sequencer[27].

RESULTS

Effect of heat shock on cell morphology of C6 cells

Heat shock caused morphological changes in C6 cells. At 37℃, the cells adhered to the culture dish and showed a normal appearance including several extrusions. At 44℃ for 30 min, the cells were still adherent, but extrusions became shorter, approximately 35 %

of the cells were dead. After shifting the cells to 48℃ for 30 min, the cells became round and possessed only a few extrusions. More than 50 % of the cells were now dead. At 52℃ for 30 min, nearly all cells were dead (> 90 %), became round and remained in suspension (Fig 1). However, during recovery for 24 h after heat shock treatment (44℃ for 30 min), some rounded cells few extrusions and re-adhered to the dish bottom. After 72 h recovery time, more and more cells assumed normal morphology (Fig 2).

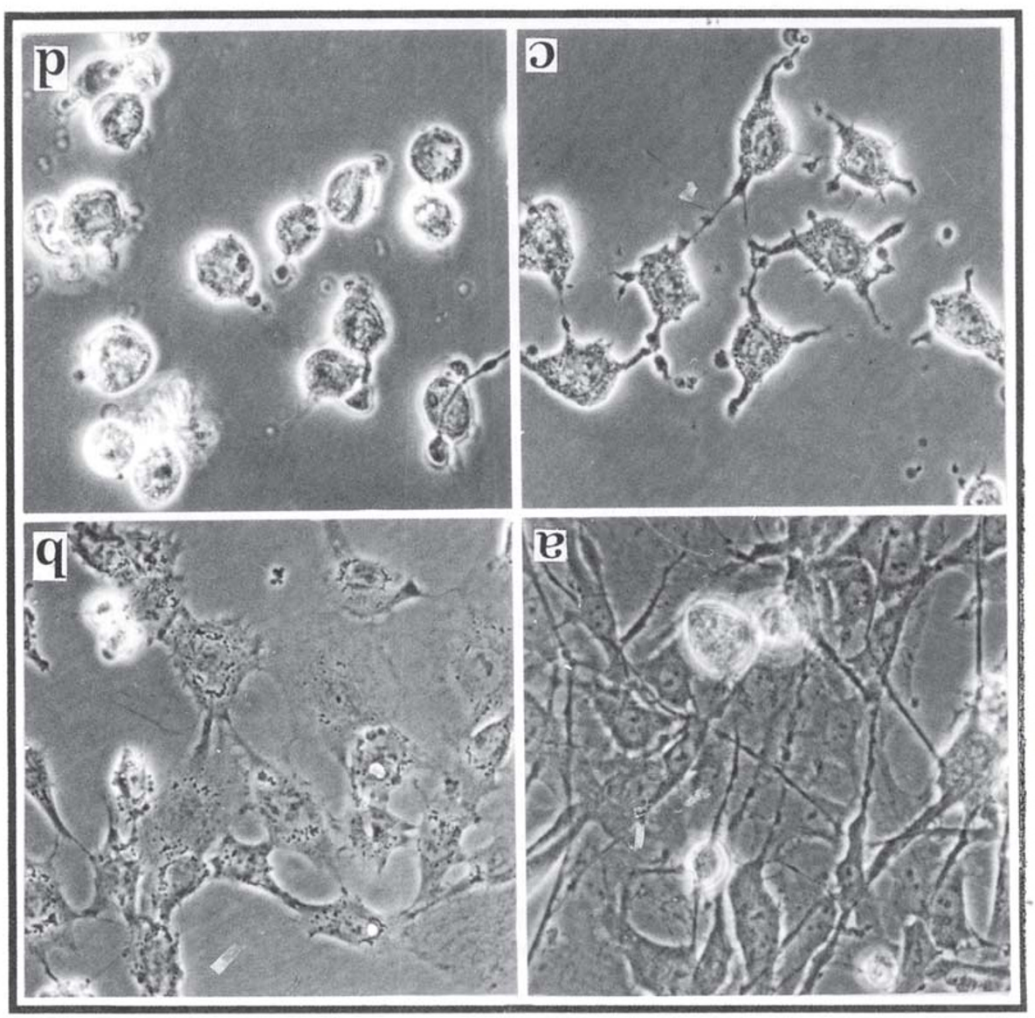

Fig 1.
Morphological change of C6 cells by hyperthermy.
The C6 cells were treated at elevated temperatures for 30 min and then observed with inverted phase contrast microscopy (400 ×) **a**. 37℃; **b**. 44℃; **c**. 48℃; **d**. 52℃.

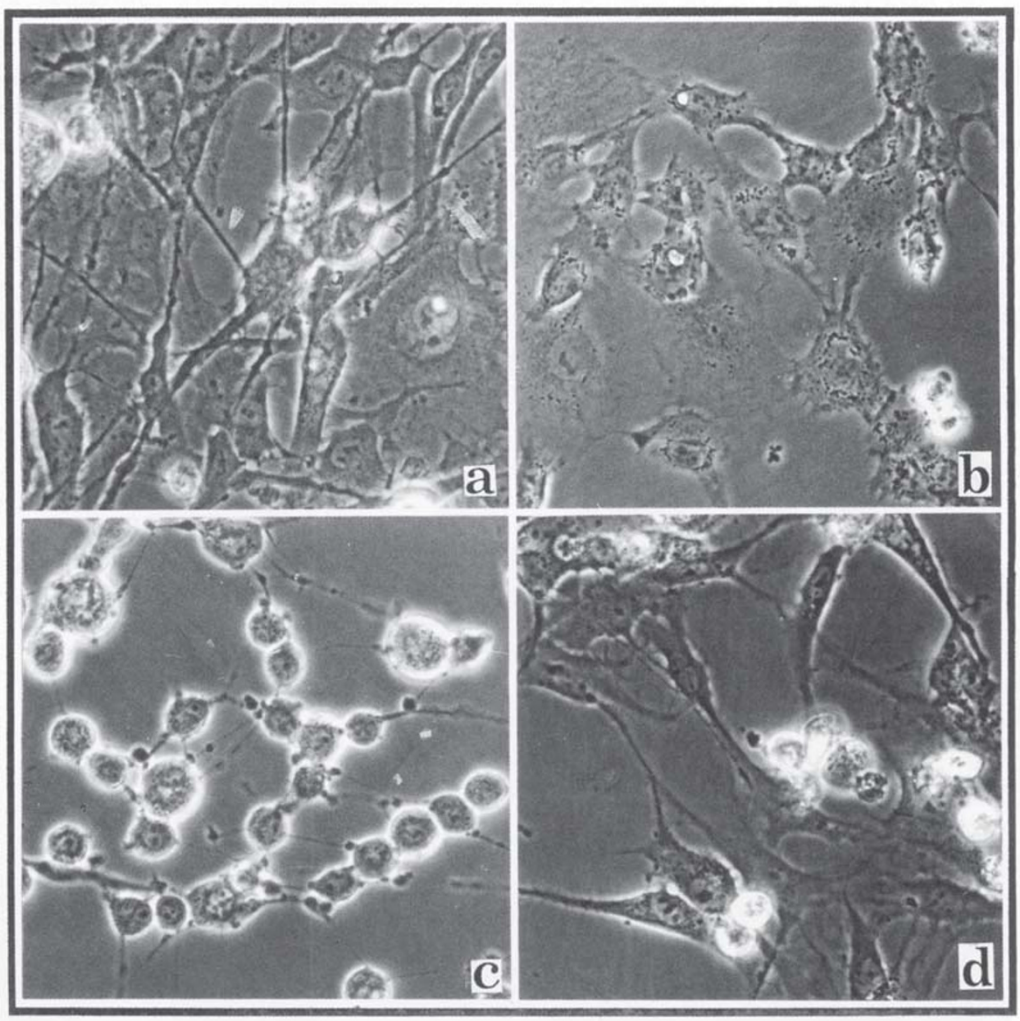

Fig 2.
Morphological changes of the C6 cells during different recovery time after heat shock (44℃ for 30 min). **a.** Control; **b.** Without recovery; **c.** Recovery for 24 h; **d.** Recovery for 72 h (400 ×).

Characteristics of ATP-BPases analyzed by G-PAGE

The results showed that the total activity of the ATP-BPases purified from the heat-shocked C6 cells is maximal at pH 8.5 when serum albumin was used as a substrate (Fig 3). Maximal activity of the 45, 55, 65 and 100 kDa proteinases was observed at pH 8.5, 8. 0, 8.0 and 10.0 respectively (Fig 3).

Characterizing the activities of these proteinases in more detail by G-PAGE showed that three bands with proteinase activity consistently appeared at 45, 55 and 100 kDa

(Fig 4a) in the control cells and that four bands with proteinase activity consistently appeared at 45, 55, 65 and 100 kDa (Fig 4b) in the heat-shocked cells. To further characterize the propeties of these proteinases, the purified proteinases were subjected to various treatments. Addition of 5 M urea to the purified proteinases before G-PAGE led to the complete abolition of activities at 45, 55 and 65 kDa, and two new bands of proteinase activities appeared at 30 and 31 kDa (Fig 4d), but b-mercaptoethanol had no such effect. The 45, 55 and 65 kDa proteinase activities were completely inhibited when the gels were incubated for 24 h at pH 8.5 in a buffer containing 10 m*M* EDTA-Na_2 (Fig 4c) or 2 μg a1-antitrypsin (Sigma) (picture not provided for being completely black as Fig 4c), which are irreversible inhibitors of metal proteinases and serine proteinases respectively. Other proteinase inhibitors, such as 2 μM pepstatin and 0.2 m*M* hydroxymercuribenzoate (HMB) had no effect on the proteinase activities in the gelatin gels (picture not provided for being the same as the control, Fig 4a).

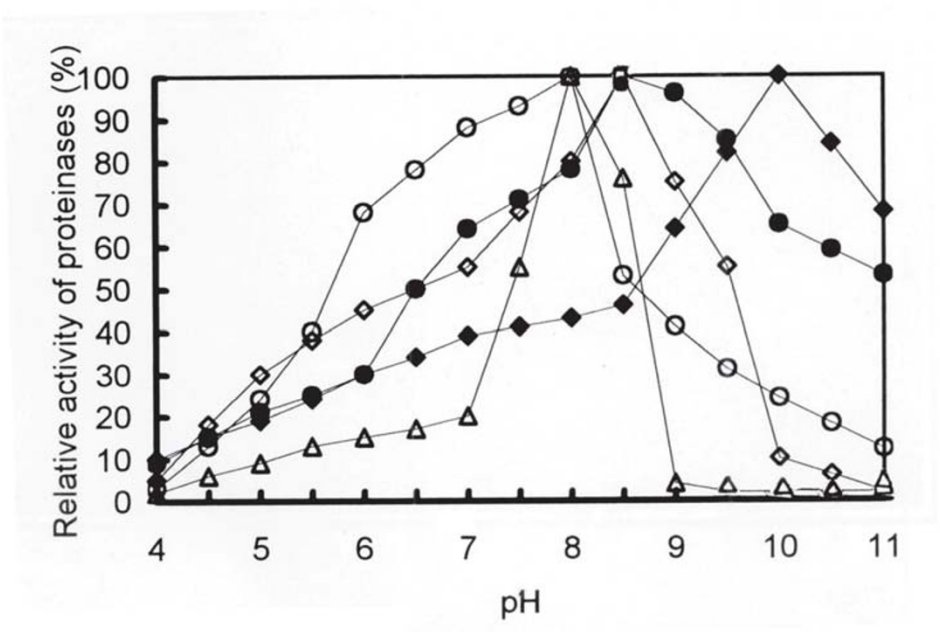

Fig 3.
pH-dependency of proteinase activities.
Total activity of the ATP-BPases was assayed using serum albumin as substrate, but individual proteinase activities were determined densitometrically. ● Total activity of the ATP-BPases; ◇ 45 kDa proteinase;o 55 kDa proteinase; △ 65 kDa proteinase; ◆ 100 kDa proteinase.

The isoelectric point (pI) of the individual proteinases of the heat-shocked (44℃ for 30 min, recovery for 12 h) and control C6 cells was determined by 2D-G-PAGE. After the separation of the proteins, the two-dimensional gelatin gels were incubated at pH 8.5 (37 ℃ for 24 h). The spots with proteinase activity showed the following pI: the 45 kDa proteinases approximately 7.1 and 4.4, the 55 kDa proteinases approximately 8.8 and 7.

1, and the 100 kDa proteinase approximately 4.9. A new appearing 250 kDa proteinase has a pI of approximately 8.5 (Fig 5) and the pI of the heat-induced 65 kDa ATP-BPase is about 5.8 (picture not provided because the activity of 65 kDa ATP-BPase was very low under these conditions).

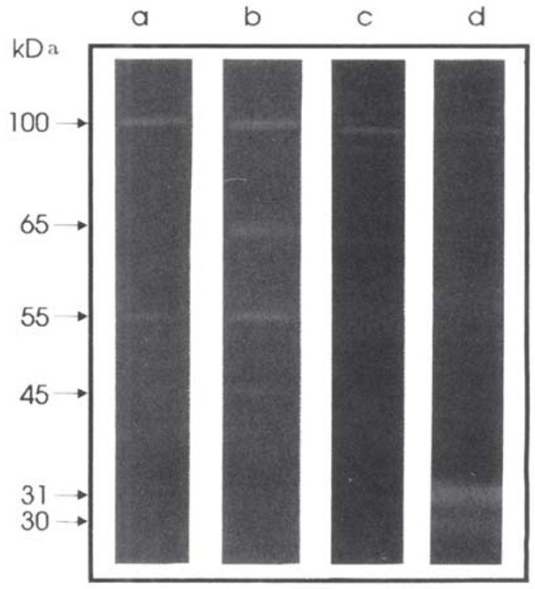

Fig 4.
Effects of different factors on the ATP-BPase activities of C6 cells (by G-PAGE, the gels were incubated at pH 8.5).
a. Control; **b.** Proteinases of the heat-shocked (44 ℃ for 30 min, recovery for 12 h); **c.** Same as b, but 10 mM EDTA was added to incubation buffer of gels; **d.** Same as b, but before G-PAGE, the proteinases were incubated in 5 M urea at 37 ℃ for 10 min.

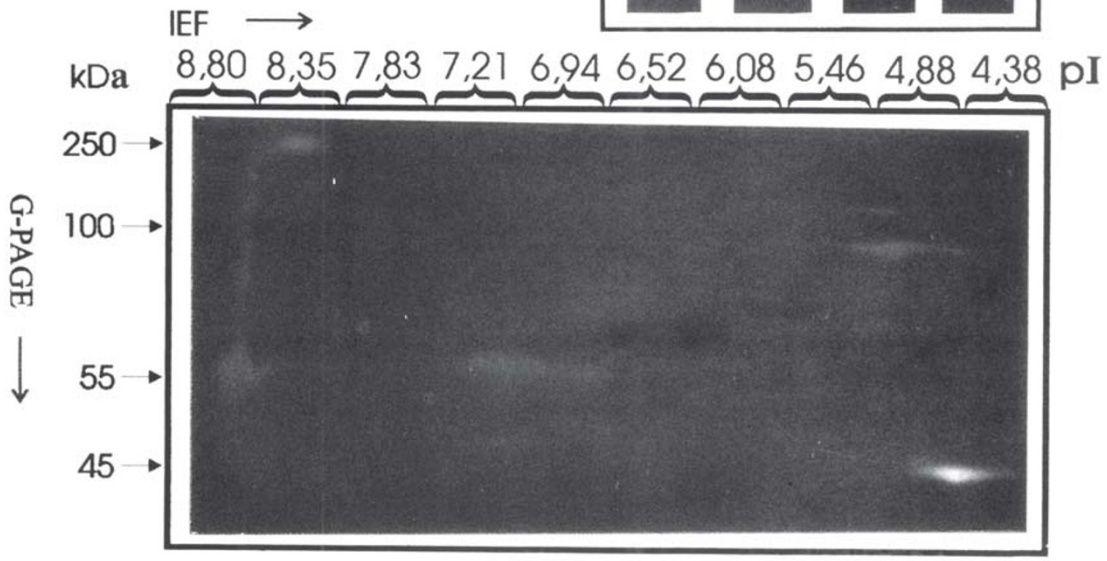

Fig 5.
pI of the purified ATP-BPases from control C6 cells (by 2D-G-PAGE, the gel was incubated at pH 8.5). Abscissa: pH scale of isoelectric focussing. Ordinate: molecular masses.

Analysis of N-terminal 1-9 amino acid sequence of the 65 kDa ATP-BPase

The 65 kDa ATP-BPase induced by heat shock (44℃) was analyzed with a Knauer protein sequencer. The results showed that its N-terminal 1-9 amino acids are

YTAMLAFLV. Amino acid hydrolysis revealed that the proteinase contained mainly hydrophobic amino acids, which accounted for 80 percent of the total amino acids. Among them, alanine, valine and leucine had a high proportion, although the most abundant amino acid was glutamic acid (Fig 6).

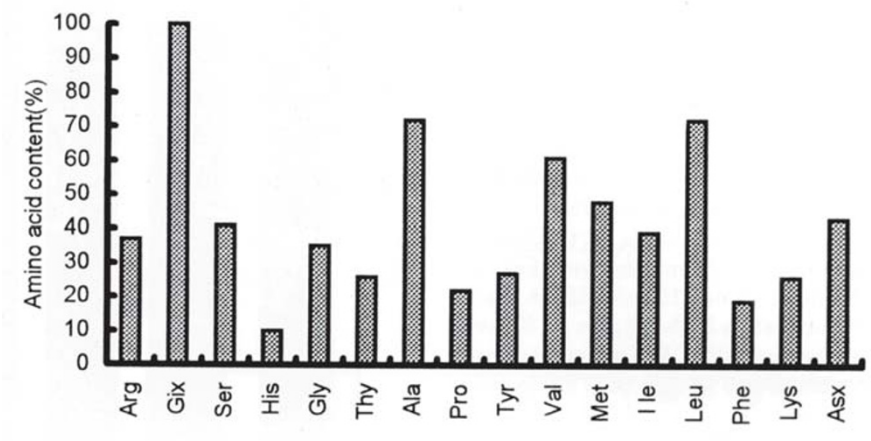

Fig6.
The total amino acid composition of the heat-induced 65 kDa ATP-BPase.

DISCUSSION

It was shown in this study that C6 cells can be induced to express a 65 kDa ATP-BPase by heat shock. The studies on its pH optimum of activity and effect of inhibitors showed that this 65 kDa ATP-BPase represented a cytosolic alkaline ATP-binding serine proteinase. Analyzing the proteinase by the Knauer protein sequencer showed that it contained 18 kinds of amino acids and its N-terminal 1-9 amino acids were YTAMLAFLV. A comparison of the N-terminal amino acid sequence and the total amino acid contents of this proteinase with protein data bases revealed no homology to other known proteinases. A comparison of the proteinase with the heat-induced sulfate proteinases in tomato[28], cathepsin B in CHO cells[17], as well as other heat-induced proteinases[7],[10],[11], showed that the heat-induced 65 kDa ATP-BPase of C6 cells has no similarity with them in their N-terminal 1-9 amino acid sequence, pH optimum, molecular weight and the effect of inhibitors. But it was reported that the heat shock (44 ℃ for 1 h and recovery for 1 h) could induce Neurospora crassa to express a 65 kDa ATP-BPase[20]. Its heat inducibility and other characteristics are similar to the 65 kDa ATP-BPase in C6 cells, but the exact relation of these two ATP-BPases is not known.

Normal C6 cells contained 45, 55 and 100 kDa ATP-BPases. Concluded from their location in the cells and optimum-pH of activity, they were very different from the re-

ported lysosomal proteinases[7] and calpain[6]. However, a comparison of properties of the 65 kDa ATP-BPase, with the 45, 55 and 100 kDa ATP-BPases in C6 cells showed that, apart from their difference of molecular masses and time of expression in heat shock response of the C6 cells (unpublished data), they are similar in the following aspects: they all can be inhibited by serine proteinase inhibitor a1-antitrypsin, dissociated by ATP-affinity chromatography and activated by Mg^{2+} and ATP; their content in the C6 cells is rather low and their maxinal activity around pH $\geqslant$ 8.0. Furthermore, it was also mentioned earlier that the 45, 55 and 65 kDs ATP-BPases all can be cleaved into 30 and 31 kDa proteinases by 5 M urea, dissociating salt- and/or ester-bonds, but β-mercaptoethanol which can dissociate disulfide bond has no such action. A comparison of these 30 and 31 kDa proteinases with the ATP-DPases, such as 26 S proteasome, 20 S proteasome, multipain, La and Ti in prokaryotes and eukaryotes, found that they were similar in consisting of same and/or different subunits[9],[14],[29], for example 20 S proteasome is comprised of twenty-eight subunits, each with molecular weight of 26 $\pm$ 6 kDa. They have serine proteinase activity, can bind with ATP and can be induced by heat shock. It would be interesting to see whether these 45, 55 and 65 kDa ATP-BPases represent precursors of the 30 and 31 kDa proteinases held together by salt- and/or ester-bonds and have certain relationship with the ATP-DPases and their subunits.

In short, the 65 kDa ATP-BPase of C6 cells as described in the present work is a new proteinase, but further studies of its aspects of structure, function, expression regulation and mechanism of action are in progress.

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