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Effects of Sucrose Concentration during Citric Acid Accumulation by *Aspergillus niger*

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A number of regulatory mechanisms are involved in increasing citric acid production under certain conditions. The accumulation of citric acid takes place under the high concentration of carbon sources. The effect of sucrose as a carbon source on the accumulation of citric acid and tyrosine phosphorylated proteins during high glycolytic conditions was investigated using *Aspergillus niger* ATCC 11414. When mycelia are treated by different sucrose concentrations, a protein of ∼27 kDa becomes tyrosine phosphorylated. This tyrosine phosphorylation takes place in response to signal transduction from the extracellular milieu. A possible role of this ∼27 kDa protein in signal transduction was demonstrated in the strain *A. niger* ATCC 11414 during cultivation on different concentrations of sucrose.

Key Words: *Aspergillus niger*, citric acid accumulation, osmotic stress, phosphotyrosine, signal transduction.

Introduction

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) has many applications in food, pharmaceutical and cosmetic industries as an acidulant, flavour enhancer, preservative, antioxidant, stabiliser, emulsifier and chelating agent[1]. It is one of the most important chemicals produced by industrial fermentation using a filamentous fungus. Most of the citric acid used in foods is derived from carbohydrate fermentation by *Aspergillus niger*[2,3].

Citric acid accumulation in substantial amounts both depends on the strain as well as on certain environmental parameters. Citric acid is accumulated when several nutrient factors are present[4−9]. One of these factors is the concentration and type of sugar[10−14]. *A. niger* is capable of producing very high levels of citric acid, about 90% of the theoretical yield from a carbohydrate source[15,16]. A high rate of acidogenesis in *A. niger* is observed only under conditions of high glycolytic metabolism[13] and can be induced by the addition of an excess amount of sucrose or other carbohydrates which induce a high rate of glycolytic catabolism. Regulation of the citric acid production metabolism takes place at several levels such as control of gene expression, translational control, control of enzyme activity and control through the

spatial distribution of enzymes[17−21]. A number of the enzymes involved in hexose utilisation are regulated by inhibitors, activators and protein phosphorylation or dephosphorylation.

When external osmolarity increases, many eukaryotic cells are capable of osmoregulation by increasing their internal osmolarity. The yeast *Saccharomyces cerevisiae* responds to increases in external osmolarity by increasing glycerol synthesis and decreasing glycerol permeability, thereby accumulating cytoplasmic glycerol up to molar concentrations[22]. Eukaryotic and prokaryotic cells develop a variety of response mechanisms to combat the harmful effects of a variety of stress conditions. This response is activated by several interacting signalling pathways controlled by protein phosphorylation. These signals are transmitted via phosphorylation and include the stress activated protein kinases[23−25].

Citric acid accumulation is known to be stimulated by high sugar concentration suggesting it may be influenced by osmotic stress. A possible role of protein phosphorylation due to osmotic stress has not been investigated in *A. niger* under citric acid production conditions. The goal of this study was to investigate the phosphorylated proteins produced during osmotic stress conditions due to high sucrose concentrations.

Experimental

Culture conditions

A. niger ATCC 11414 was used as a citric acid producer throughout this work. Stock cultures were kept at 4 °C on malt-agar slants and subcultured monthly. Conidia of 5-to-7-days old cultures on malt-agar slants incubated at 30 °C were used for the preparation of inocula. Two percent of a conidial suspension in sterile NaCl solution, containing 5 x 10^8 mL^{-1} of conidia, was added to the sterile medium. The medium used was based on that described by Shu and Johnson[26] and contained (g/L): decationised sugar 140; NH_4NO_3, 2.5; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.25; Fe^{3+} [as $(NH_4)_2SO_4.Fe_2(SO_4)_2.24\ H_2O$], 0.1 x 10^{-3}; Zn^{2+} (as $ZnSO_4.7H_2O$), 0.1 x 10^{-3}; and Cu^{2+} (as $CuSO_4.5H_2O$), 0.06 x 10^{-3}. pH was adjusted to 3.0 with 1 N HCl. For cultivation on different concentrations of sucrose, the 20% (w/v), decationised sucrose stock solution was diluted with double distilled water to the desired final concentration. Sucrose was decationised on a Dowex W-50AG 1-X8 before use[19]. All media were autoclaved at 121 °C for 20 min. The experiments were carried out in 1 L wide-mouthed shaken flasks, containing 150 mL of medium, on a rotary shaker (360 rpm) at a temperature of 30 °C.

Preparation of cell free extracts

Cell free extracts were prepared from freshly harvested mycelia by a procedure described in the literature[27], which involves grinding under liquid nitrogen and subsequent ultrasonication in a Bandelin-Sonopuls HD 60 ultrasonifier for 30 s. The extractant was 20 mM Tris/HCl, pH 7.5, containing 2 mM EDTA and 5 mM 2-mercaptoethanol. The homogenate was centrifuged at 10,000 x g (20 min, 4 °C) and the clear supernatant kept at –20 °C until use.

Analytical methods

The phenol-sulphuric acid method was used for the quantitative determination of sugars in the culture fluid[28]. Quantification of citric acid was done by enzymatic analysis using a commercial test kit (Boehringer-Mannheim). The protein concentration of cell free extracts was determined by the Coomassie Blue method[29].

Electrophoresis

Protein electrophoresis was performed according to Laemmli[30]. Samples were mixed with equal volumes of sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled (3 min), and subjected to vertical slab-gel SDS-PAGE, using 12% gels. Proteins were either stained by Coomassie Brilliant Blue R-250 solution (60 min, room temperature) or transferred onto a membrane for immunoblotting.

Immunoblotting

After electrophoresis, proteins were transferred from the gel to nitro-cellulose membranes in transfer buffer [25 mM Tris base, 190 mM glycine, 20% (v/v) methanol, pH 9.6]. Blots were washed in Tris-buffered saline-Tween 20 (TBS-T), incubated with the desired antibody and then probed with the appropriate horseradish peroxidase (HRP)-conjugated antibody. The enhanced chemiluminescence (ECL) method was utilised for detection.

Immunoaffinity chromatography

Isolation of tyrosine phosphorylated stress-active proteins from cell free extract was carried out by immunoaffinity chromatography on a column of monoclonal anti-phosphotyrosine-agarose and equilibrated in buffer A (20 mM Tris/HCl, pH 7.5, containing 2 mM EDTA, 0.5 M NaCl and 5 mM β-mercaptoethanol). The specific protein bonded was eluted with elution buffer (0.1 M citric acid).

Results and Discussion

Effect of sugar concentration

The yield of citric acid accumulated by *A. niger* increases by cultivation on increasing concentrations of sucrose. In order to see whether this correlation also occurs with the strain of *A. niger* used in this work and to understand whether this phenomenon occurs throughout the time of incubation, the fungus was pregrown on a medium containing 1% (w/v) sucrose for 48 h. After this, mycelia were transferred to new media containing 14% or 5% (w/v) sucrose, respectively. Citric acid concentration was determined at 24 h intervals throughout cultivation.

As shown in Figure 1, the accumulation of citric acid increased considerably after 48 h for 14% sucrose. The rise continued up to 120 h and then slowed down. Seventy-six percent of the sucrose initially present was converted to citric acid at 216 h of incubation. In contrast, on a medium containing 5% (w/v) sucrose, the accumulation of citric acid occurred at a much lower rate, and commenced only until 72 h.

The results from this experiment and previous studies show that sucrose concentration exerts its effect particularly during the early stages of cultivation after replacement[12,21,31–36]. All further experiments were therefore performed within the first 72 h of incubation after replacement. Thus, *A. niger* was grown on precultivation medium and then placed into different concentrations of sucrose [14%, 10%, 7.5%, 5% and 2.5% (w/v)] as stress conditions. Cell free extracts were prepared from mycelia incubated under these conditions, and the presence of tyrosine-phosphorylated proteins analysed by immunoblotting and immunostaining using monoclonal phosphotyrosine antibodies. Certain culture parameters, such as citric

acid concentration, growth rate, sugar consumption and product yield were also determined for 24, 48 and 72 h of incubation.

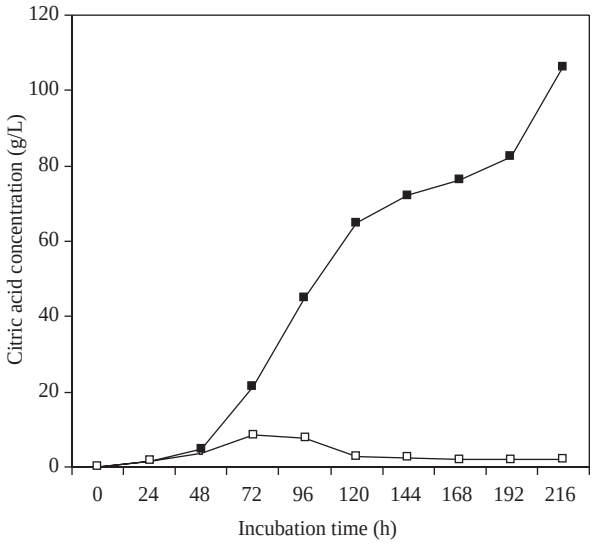

Figure 1. Changes in citric acid concentration (g/L) during growth of *A. niger* upon placement into medium containing 14% (w/v) and 5% (w/v) sucrose. Key to symbols: ■ = 14%, □ = 5% (w/v) sucrose.

Changes in citric acid concentration, mycelial growth and sugar consumption during placement in different sucrose concentrations

The effect of different sucrose concentrations on citric acid production, mycelial growth and sugar consumption is shown in Figures 2a-e.

As can be seen in Figure 2a, there is a linear relationship between the sucrose concentration initially present and the intracellular amount of citric acid accumulated for the medium which contained 14% (w/v) sucrose. Citric acid accumulation increased with consumed sucrose concentration. This increase was also accompanied by biomass formation. The rate of accumulation changed significantly after 48 h. However, (Figure 2b) the highest rate of citric acid accumulation was obtained during growth on the medium containing 10% (w/v) sucrose. Mycelial growth rates showed gradual increases and did not accompany acid accumulation. The sugar concentration decreased suddenly and reached half of its initial amount after 24 h. Citric acid accumulation increased parallel to initial sucrose concentration in media containing 7.5% (w/v) sucrose. Mycelial growth increased over time (Figure 2c), and was independent of initial sucrose concentration and the optimal rate was determined. Lower rates of acid accumulation were recorded under growth on 5% (w/v) sucrose (Figure 2d). Citric acid accumulation slowly increased until 48 h during growth on 2.5% (w/v) sucrose, but then remained constant during further fermentation time because all the sucrose was utilised (Figure 2e).

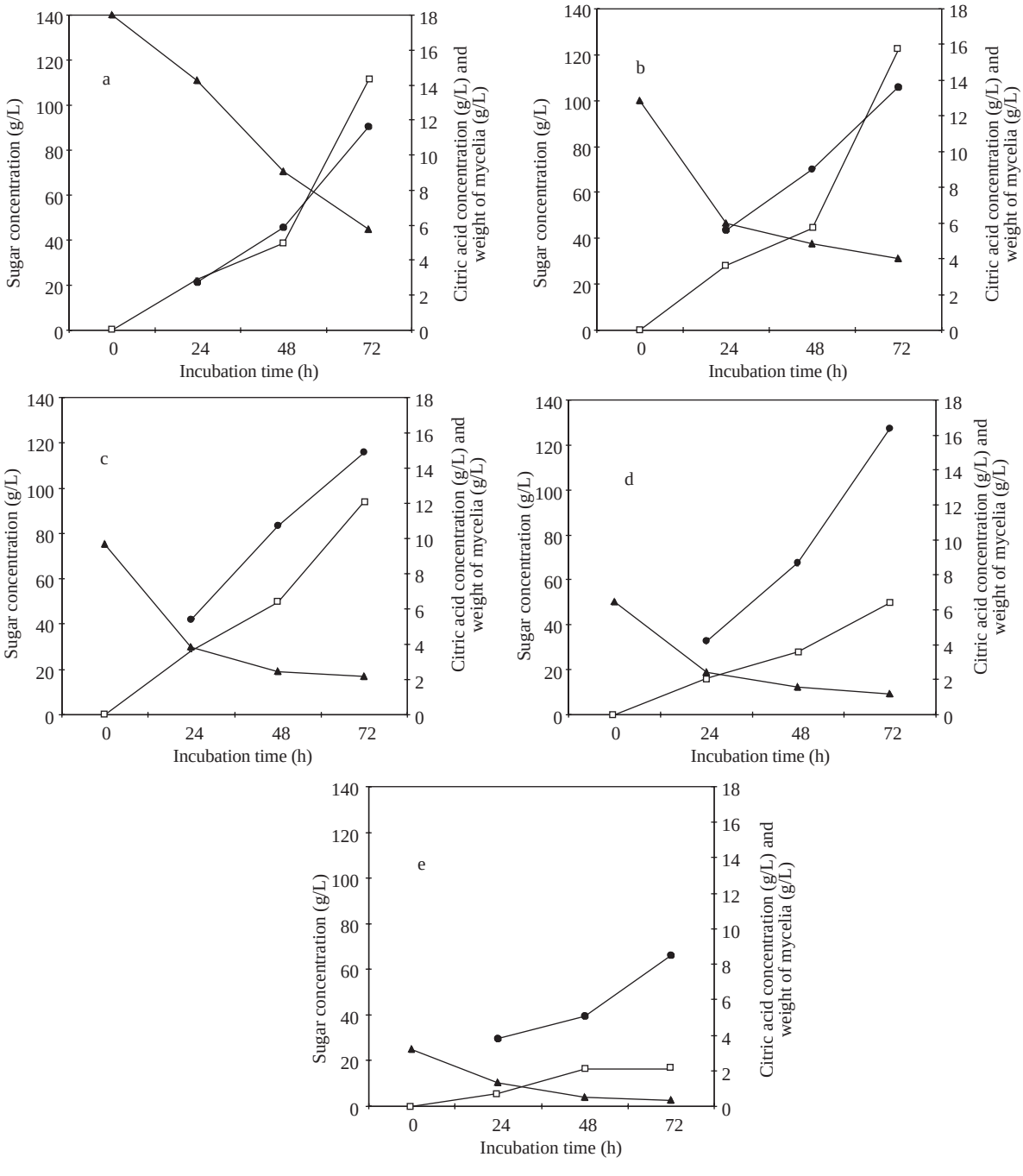

Figure 2a-e. Changes in citric acid concentration (g/L), mycelial biomass (g/L) and sugar concentration (g/L) during growth on different concentrations of sucrose; [(a) = 14% (w/v), (b) = 10% (w/v), (c) = 7.5% (w/v), (d) = 5% (w/v), (e) = 2.5% (w/v)] Key to symbols: ▲ = Sugar □ = Citric acid ● = Biomass.

Basically, an increase in citric acid accumulation with increasing concentrations of sugar is not surprising as it may simply reflect the availability of more substrate. In order to learn whether this is also due to a metabolic effect, the yields of citric acid ($Y_{p/s}$) were compared. Figure 3 shows that a high yield was characteristically obtained only at concentrations of sucrose lower than 10%. Growth at sucrose concentrations higher than 10% caused a decrease in $Y_{p/s}$ values.

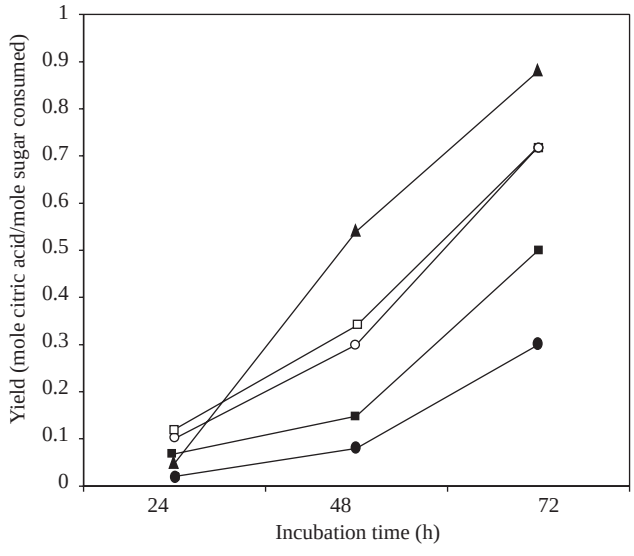

Figure 3. Changes in the yield (mole citric acid produced/mole sugar consumed) during growth on different concentrations of sucrose; Key to symbols: ● = 14%, ■ = 10%, □ = 7.5%, o = 5%, ▲ = 2.5% (w/v) sucrose.

A different picture was obtained, however, when the specific concentration was compared (Figure 4): the amount of acid accumulated per gram of mycelium was highest for 14%, 10% and 7.5% sucrose, and gradually decreased until 2.5% which showed the lowest concentration per gram of biomass.

From this data it seems clear that sucrose concentration affects citric acid production in the strain of *A. niger* used.

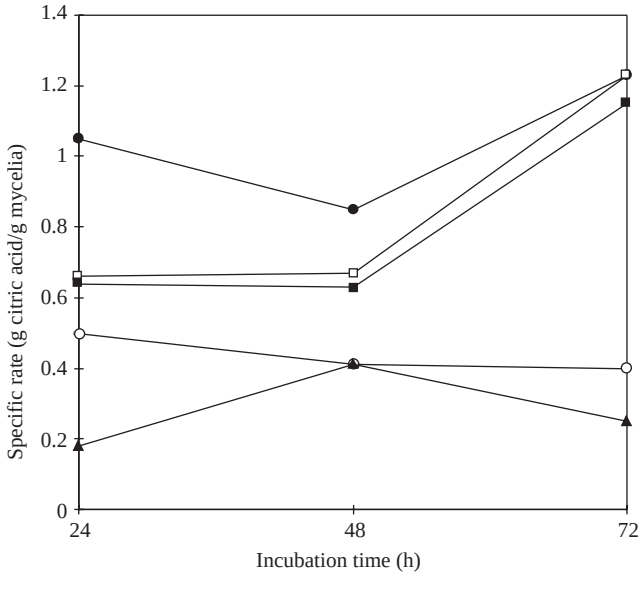

Figure 4. Citric acid formation per gram of mycelia for different concentrations of sucrose. Key to symbols: ● = 14%, ■ = 10%, □ = 7.5%, o = 5%, ▲ = 2.5% (w/v) sucrose.

Tyrosine-phosphorylated proteins under different concentrations of sucrose

In order to see if tyrosine phosphorylation occurs in response to osmotic stress conditions on *A. niger* during citric acid accumulation, the fungus was cultivated on different concentrations of sucrose (14%, 10%, 7.5%, 5%, and 2.5%; w/v) as stress conditions.

The result from an immunoblot analysis of tyrosine-phosphorylated proteins is shown in Figure 5. This data showed that approximately 20 kDa, 27 kDa, 50 kDa and 85 kDa proteins were phosphorylated on tyrosine residue. As a response mechanism to osmotic stress, Tyr-phosphorylated major protein 27 kDa was obtained during overall fermentation time. However, the specific protein disappeared after 24 h during growth on 14% (w/v) sucrose. The medium which contained 10% and 7.5% sucrose exhibited Tyr-phosphorylated protein during overall fermentation time. Specific protein disappeared after 48 h during growth on 5% (w/v) and 2.5% (w/v) sucrose. From these results, it is seen that *A. niger* responds to the osmotic effect inducing the activation of an osmosensing signal transduction pathway which activates the 27 kDa protein.

However, an approximately 20 kDa Tyr-phosphorylated protein was obtained during growth on high concentrations of sucrose ($\geq$ 7.5). Only growth on 14% (w/v) sucrose exhibited Tyr-phosphorylated protein at 85 kDa. Growth on 10% (w/v) and 7.5% (w/v) sucrose showed this protein at an early stage of fermentation (24 h). This 85 kDa protein can be an active phosphorylated form of 6-phosphofructo-1-kinase (PFK1) (native PFK1 is 84 kDa[31]) due to high concentrations of sugar. The 50 kDa Tyr-phosphorylated protein was obtained during growth on 14% (w/v) and 10% (w/v) sucrose at 24 and 48 h of incubation time.

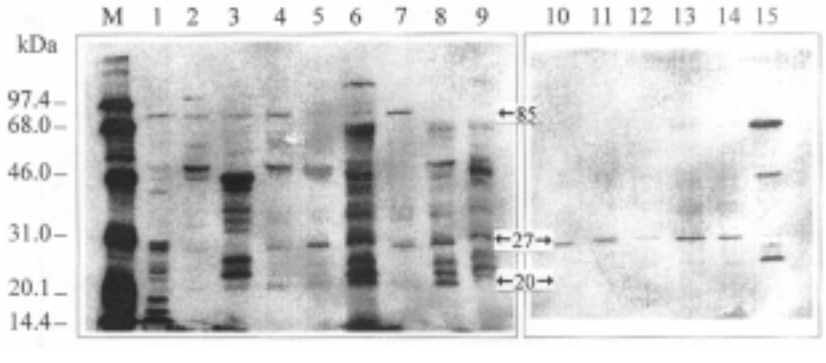

Figure 5. Immunoblot analysis of phosphotyrosine proteins in cell free extracts of *A. niger*. M: Low molecular weight marker, (fractionated proteins from different concentrations of sucrose for 24 h, 48 h and 72 h of incubation) Lane 1-3: 14%, Lane 4-6: 10%, Lane 7-9: 7.5%, Lane 10-12: 5% and Lane 13-15: 2.5% sucrose (w/v).

In order to prove whether this 27 kDa protein is activated by Tyr-specific protein kinases, *A. niger* was cultivated in media containing different concentrations of sucrose and the tyrosine kinase inhibitor, genisteine (4',5,7-Trihydroxyisoflavone). Citric acid determinations were performed at daily intervals. No effects were obtained for citric acid accumulation during growth on media containing the inhibitor. The accumulation of Tyr-phosphorylated proteins was completely prevented by the inhibitor.

Characterisation of 27 kDa tyrosine-phosphorylated protein

To purify the major tyrosine phosphorylated 27 kDa protein, immunoaffinity chromatography with monoclonal anti-phosphotyrosine agarose purified mouse antibody was used.

In order to confirm that this protein is induced tyrosine phosphorylated, immunoblot analysis of the purified fraction was also performed. The results are shown in Figure 6.

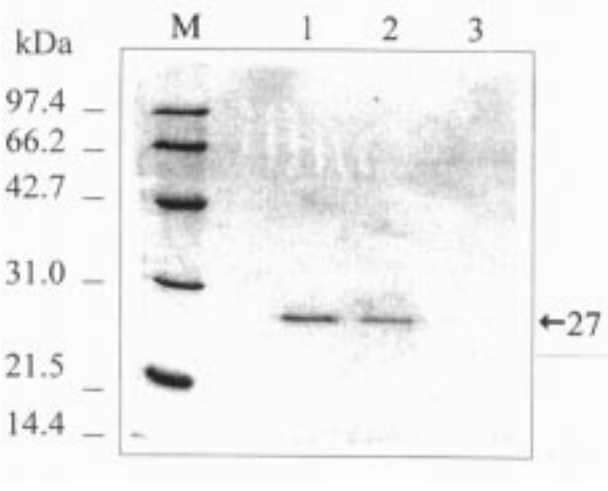

Figure 6. Immunoblot analysis in the presence of primary anti-phosphotyrosine antibody. M: ECL low molecular weight marker, Lane 1-5: 27 kDa Tyr-phosphorylated protein.

Conclusions

The effect of osmotic stress dependence on sucrose concentrations and the production of osmosensing proteins were investigated during citric acid accumulation by *A. niger* ATCC 11414. The results demonstrated that sucrose concentration affects the citric acid production of the present strain and high sugar dependent osmotic stress induced the tyrosine phosphorylation of the approximate 20, 27, 50 and 85 kDa proteins. The most prominent response occurred in the 27 kDa (p27) band. A major tyrosine phosphorylated p27 was purified. The 85 kDa phosphorylated protein could be the active form of PFK1 whose native is 84 kDa[37]. Kubicek-Pranz et al.[12] found that triggering citric acid accumulation by placing *A. niger* onto high concentrations of sucrose or glucose is paralleled by a rise in the intracellular concentration of Fructose-2,6-diphosphate (Fru-2,6-P_2), the strongest activator of PFK1[33]. Higher concentrations of Fru-2,6-P_2were observed in mycelia cultivated on sucrose, which allows higher yields of citric acid to be obtained[33]. The regulatory osmotic effect of high sugar concentration on citric acid accumulation is due to a possible role of tyrosine kinases in regulating the ion efflux pathways induced by hyper-osmotic stimulation. These kinases are required for restoring the osmotic gradient across the cell membrane in response to increased external osmolarity[38−39].

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