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Abstract

Attempts have been made earlier to understand the mechanisms of osmoregulation and the ability ot adapt to fluctuations in the external osmolarity (Ben-Amotz and Avron 1983. Ann. Rev. Microbiol. 37:95-119, Csonka 1989. Microbiol. Rev. 53:121-147). Various strategies have been adopted by the cells in order to survive and proliferate in the presence of reduced water activity, including accumulation of carbohydrates. amino acids and quaternary ammonium compounds (Measures 1975. Nature 257:398-400, Brown et al. 1972. J. Gen. Microbiol. 72:589-591, Dannibier et al. 1988. Arch. Microbiol. 150:348-357). In the present study, attempts were made to investigate the role of amino acids that accumulate intracellularly in halotolerant Aspergillus repens under salt stress condition.

Role of amino acids in halotolerant *Aspergillus repens*

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Attempts have been made earlier to understand the mechanisms of osmoregulation and the ability ot adapt to fluctuations in the external osmolarity (Ben-Amotz and Avron 1983. Ann. Rev. Microbiol. 37:95-119, Csonka 1989. Microbiol. Rev. 53:121-147). Various strategies have been adopted by the cells in order to survive and proliferate in the presence of reduced water activity, including accumulation of carbohydrates. amino acids and quaternary ammonium compounds (Measures 1975. Nature 257:398-400, Brown et al. 1972. J. Gen. Microbiol. 72:589-591, Dannibier et al. 1988. Arch. Microbiol. 150:348-357). In the present study, attempts were made to investigate the role of amino acids that accumulate intracellularly in halotolerant Aspergillus repens under salt stress condition.

Aspergillus repens was grown in 50 ml synthetic medium in 250 ml Erlenmeyer flasks. The composition and culture condition was essentially the same as described earlier (Parekh and Chhatpar 1989. Curr. Microbiol. 19:297-301). For amino acid analysis, cell-free extracts were treated with trichloroacetic acid (TCA). After precipitation and centrifugation, TCA was removed from the supernatant by chilled supernatant. The supernatant was then subjected to amino acid analysis by automatic amino acid analyser (LKB) with known standards. Proteases were assayed as described earlier (Chhatpar et al. 1984. Experientia 40:1382-1384) from the cell-free extracts. Glucose-6-phosphate dehydrogenase (G6PDH) and FDP aldolase activity was assayed as described by Chhatpar et al. (op. cit.). Glutamate dehydrogenase (GDH) activity was assayed using the procedure described by Meers and Tempest (1970. J. Gen. Microbiol. 64:187-190).

Significant differences were observed in free amino acid pools at 144 h when the mold was grown in medium supplemented with 2 M NaCl (stress condition) as compared to control growth conditions (Table 1). At 72 hours, however, no or few amino acids were observed. Amino acids like porline, serine and glutamate were found to be higher in mold grown under stress as compared to control conditions. Non-polar amino acids like alanine, valine and leucine were found to be significantly higher than other free amino acids detected. When the osmolytes like glutamate, proline, glycerol and glycine-betaine were added to the growth medium at a concentration of 10 mM, there was an enhanced production of free amino acids (Table 1) under saline conditions.

Analysis of intracellular proteases (acidic, neutral and alkaline, pH optima 5.5, 7.0 and 8.6, respectively) revealed an increase in their activities at all stages of growth (Figure 1) under stress as compared to controls. The intracellualr portease activity was significantly higher from 48 to 72 h and thereafter gradually decreased. Other enzymes like FDP aldolase and GDH were also more active uncer stress as compared to controls at 72 and 144 h of growth (Table 2).

An increase in the amino acid pool as well as an increase in specific amino acids will act in several ways to overcome salt stress problems. They are listed as follows: a) Glycine and alanine have high water solubility and will rescue the cell by overcoming reduced water activity; b)

Proline associates itself with its hydrophobic part with the hydrophobic side chain of protein, thereby converting them into hydrophilic groups by exposing the carboxylic and imino groups towards the water molecules providing a proper water structure under reduced water activity (aw); c) Acidic amino acids like glutamate and apsartate have a net negative charge. It is possible that internally accumulated acidic amino acids will sequester sodium and will diminish the excessive positive Na+ charge; d) Osmotic accumulation of K+ is regulated by proline; e) Glycine-betaine and glutamic acid increase with the increasing external osmotic strength and overcome growth inhibition caused by osmotic stress by protecting enzymes from inactivation at high ionic strength.

Decreased levels of free amino acids at 72 h may be accounted for by their rapid utilization for the de novo synthesis of proteins rather than their production through degradation of proteins, since new proteins may be essential for growth under 72 h. However, comparatively more hydrolysis of proteins may be taking place than synthesis resulting in more amino acids accumulating at 144 h of growth under stress as compared to control. Barlow et al. (1976. Crop Sci. 16:59-62) have observed 20 t0 250% increase in free amino acids in Zea under stress. Similar results were obtained in bean leaves (Huber et al. 1977. Z. Pflanzen. Physiol. 31:234-247). The accumulation of significantly higher levels of free amino acids within the cell would draw water back into the cell and thus lower the otherwise deleterious concentrations of intracellular solutes.

In our studies there was not only a net increase in total amino acids under stress but levels of non-polar amino acids like alanine, leucine and valine were found to increase at a higher rate compared to other amino acids. Ben-Naim (1980. Plenum Publishing Corp., New York) has investigated the phenomenom of hydrophobic effect. Non-polar molecules in aqueous solution tend to aggragate, squeezing out water. Although different assemblies of water molecules have different energies, each component of the system has to contribute to the equilibrium i.e. final state, in which water must have the same chemical potential throughout. Water is in a state of low free energy because of high concentration of solutes (here, as NaCl) and thus increase its chemical potential. Water adjacent to the non-polar molecules move apart and decrease their chemical potential. It is possible that non-polar amino acids (i.e. leucine, valine and alanine) in aqueous solution function by decreasing the chemical potential, to control the chemical potential of the cell because of sodium chloride which would otherwise have been deleterious to the cell.

Protein turnover is essential for the adptation of cells to new environmental conditions, and intracellular proteases play a vital role in protein turnover processes, along with protein synthesis. A high level of protease at 48 to 72 h indicates a high protein breakdown and hence the turnover at these hours, but surprisingly, the free amino pool at this stage is very low, suggesting the possibility of more conversion to, than degradation of protein at this stage. The reverse may be true at 144 h where synthetic potential may be comparatively much less than degradation leading to accumulation of amino acids.

Besides the production of proteases, there was a significant increase in activities of enzymes like FDP aldolase, cytosolic GDH and G6PDH at 72 and 144 h in cells grown under stress as compared to controls. Increase in the activity of FDP aldolase and G6PDH under saline conditions may suggest an increase in the rate of glycolysis and hexose monophosphate shunt

(HMP shunt) which will provide intermediates for amino acid biosynthesis. The glycolytic intermediate pyruvate may provide alanine, valine and leucine while 3 P-glycerate would give rise to serine, cysteine and glycine. Phosphoenol pyruvate and erythrose 4-P may produce phenylalanine, tyrosine and tryptophan, while histidine can be produced from ribose. Saline conditions increase GDH activity, which will produce glutamate, resulting again in the production of proline and arginine. Aspratate is produced from oxaloacetate. Aspartate in turn can give rise to isoleucine, lysine and threonine. Thus, saline conditions can enhance the cellular metabolic pathways like glycolysis, TCA cycle and HMP shunt, etc. which may lead to the production of intermediates of amino acid biosynthetic pathways and thus contribute to an increase in the amino acid pool under saline conditions.

In our studies with *Aspergillus repens*, then, total amino acid accumulation, enhanced production of non-polar amino acids and increased levels of proteases and enzymes of carbohydrate metabolism may play crucial roles enabling adaptation to saline conditions.

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Figure 1. Intracellular protease activity from *Aspergillus repens* grown under saline conditions. 2M= 2 M NaCl-supplemented media; C=control medium (no NaCl)

Table 1. Amino acid analysis of free amino acid pool from *Aspergillus repens* grown in the presence and absence of osmoregulators (10 mM) at 72 and 144 h of growth:

Table 2. Enzyme activities from *Aspergillus repens* grown under control and saline conditions: