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Salt mediated changes in some enzymes of carbohydrate metabolism in halotolerant *Cladosporium sphaerospermum*

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Abstract. *Cladosporium sphaerospermum,* isolated from salt pans was halotolerant. When grown in the presence of salt, the activities of invertase, isocitrate lyase, fructose-1,6 diphosphate aldolase and malate dehydrogenase were found to be increased and that of amylase decreased. Both, enzyme activation as well as an increase in *de novo* synthesis of enzymes were found to be some of the mechanisms of salt mediated changes. This may be one of the adaptive mechanisms, in halotolerant *Cladosporium sphaerospermum.*

Keywords. *Cladosporium sphaerospermum;* halotolerant; invertase; amylase; aldolase; malate dehydrogenase; isocitrate lyase.

Introduction

The enzymes from extremely halophilic bacteria represent a fascinating example of adaptation. These enzymes perform their functions *in vivo* at 4–5 M NaCl, losing activity rapidly when exposed to low salt concentration (Lanyi, 1974). There is paucity of information regarding the salt effects in marine fungal systems as compared to bacteria.

Physiological studies on marine fungi have tended to concentrate mainly on their salinity requirements (Harrison and Jones, 1974), spore germination (Byrne and Jones, 1975), sporulation (Harrison and Jones, 1975), and some aspects of physiology and biochemistry (Jennings, 1983). Not much is known about the effect of salt on enzymes from fungal systems. The present study therefore deals with the effect of salt on some of the enzymes of carbohydrate metabolism in the fungus *Cladosporium sphaerospermum* isolated from salt pans.

Materials and methods

Isolation of fungal culture

The fungal culture was isolated by streaking the soil samples obtained beneath the salt pans on Saboraud's agar plates and incubating at 30°C till growth was obtained. The composition of Saboraud's agar used was (in grams per 100 ml): glucose 2·5; peptone 1·0; NaCl 0·5 and agar (Centron) 2·0. The pH was adjusted to 5·5. Fungal isolate was maintained on Saboraud's agar slants supplemented with 2 M NaCl.

Identification of fungal culture

The identification of fungal culture was done with the kind help of Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, England.

Composition of liquid growth medium

The composition of the liquid growth medium employed was as follows (g/litre): Yeast extract 1·0; (N H4) $_2$ SO_4 3·5; KH_2 PO_4 10; Mg SO_4 2·0; Sucrose 85; Casamino acids 1·0; $CaCl_2$ 0·075; $ZnSO_4$ 0·010; $MnCl_2$ 0·005; ammonium molybdate 0·002; $Na_2B_4O_7$ 0·002 and $FeSO_4$ 0·002. The pH was adjusted to 5·5.

For experiments on amylase production, 1 % starch was used as a carbon source in the liquid growth medium instead of sucrose.

Growth of the culture in liquid medium

The culture was grown in 50 ml liquid medium in 250 ml Erlenmeyer flasks on a rotary shaker (180 rpm) at 30°C for 72 h unless and otherwise stated. The mycelia were harvested by filtration and rapidly transferred to — 5°C before use. Cycloheximide whenever required was added at 10μg/ml.

Method for the preparation of cell free extract

For intracellular enzyme assays, a cell-free extract was prepared in 0·05 M Tris HCl buffer of pH 7·2 by grinding the frozen mycelia with a pestle in a chilled mortar with glass powder. The extract was centrifuged at 15,000 *g* for 30 min and the supernatant obtained was used for intracellular enzyme assays.

Assay method for FDP aldolase (fructose-1,6 diphosphate D-glyceraldehyde-3 phosphate lyase, EC 4.1.2.13)

FDP aldolase was assayed according to the method of Jagannathan *et al.* (1954). The unit of activity is expressed as the amount of enzyme which causes a change of 0·001 O.D. at 240 nm per min under the experimental conditions.

Assay method for malate dehydrogenase (L-malate; nicotinamide adenine dinucleotide oxidoreductase, EC 1.1.1.37)

Malate dehydrogenase was assayed as described by Ochoa (1955).

A unit of malate dehydrogenase is defined as the amount of enzyme needed to bring about decrease of 0·001 O.D. at 340 nm per min at 30°C.

Assay method for isocitrate lyase (threo-D-isocitrate glyoxylate lyase, EC 4.1.3.1)

Isocitrate lyase was assayed according to the method of Dixon and Kornberg (1959). A unit is defined as the amount of enzyme which causes an increase of 0·001 O.D. at 324 nm per min at 30°C under experimental conditions.

Assay method for invertase (ß-fructofuranoside fructohydrolase, EC 3.2.1.26)

Invertase activity was measured by estimating reducing sugars released as described by Bernfeld (1955) method. A unit is described as the amount of enzyme which causes the liberation of 1 μmol of reducing sugar at 37°C per h.

Assay method for amylase (1,4 D-glucan maltohydrolase, EC 3.2.1.2)

Amylase activity was measured according to the method of Bernfeld (1955).

A unit is described as the amount of enzyme which causes the liberation of 1 μmol of maltose at 37°C per h.

Assay method for protein

Protein was assayed according to the method of Lowry *et al.* (1951).

Results and discussion

C. sphaerospermum was isolated from salt pans. It did not show an obligatory requirement of salt for growth. The mold was thus found to be halotolerant. Mold could utilize glucose, sucrose or starch as carbon sources. Extracellular invertase activity was found to increase when sodium chloride was added to the growth medium upto 2 M concentration, while amylase activity decreased (table 1).

Table 1. Effect of sodium chloride added in the growth medium on the activities of enzymes from *C. sphaerospermum.*

NaCl (M)	Invertase	Amylase	Malate dehydrogenase	FDP aldolase	Isocitrate lyase
	Units/flask (50 ml)		(Units/mg protein)		
0·0	320	708	27	90	81
1·0	667	314	67	112	101
2·0	1372	231	65	261	244

FDP aldolase, isocitrate lyase and cytosolic malate dehydrogenase showed significantly higher activities in cultures grown in the presence of sodium chloride (table 1). Earlier, Baxter and Gibbons (1956) have shown that in certain halophilic bacteria, a number of enzymes like isocitrate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, lactate dehydrogenase and cytochrome oxidase, required very high salt concentrations for maximal enzyme activity. Larsen (1963) also showed that catalase and glutamate-aspartate transaminase required very high salt concentration for maximal activity.

In order to understand the effect of salt on enzyme activities, in *vitro* studies were carried out to see the effect of salt on kinetic constants such as K_m and $V_{max.}$ Substrate saturation curve for invertase from *C. sphaerospermum* did not appear to obey normal

Michaelis-Menten kinetics. It appears to be sigmoidal with negative co-operativity. By the addition of the modulator NaCl, both V_{max} and *Km* values were found to be increased. It therefore deviates from *'K'* or *'M'* types of enzymes. Co-operative or allosteric effects have been observed in a number of halophilic systems (Aitken *et al.*, 1970; Dundas and Halvorson, 1966; Liebl *et al.*, 1969) but the influence of salt on the regulatory properties of these enzymes has not been extensively investigated.

The effect of NaCl was also seen *in vitro* on the activity of partially purified isocitrate lyase. Here also both K_m and V_{max} values were found to be increased. Similar results were obtained with malate dehydrogenase. Isocitrate lyase and malate dehydrogenase showed normal Michaelis -Menten kinetics. Thus in all the cases *i.e.* invertase, isocitrate lyase and malate dehydrogenase, both K_m and V_{max} values were found to be increased (table 2) in the presence of sodium chloride as compared to control.

Table 2. Effect of sodium chloride on *Km* and *V*max values of invertase, isocitrate lyase and malate dehydrogenase from *C. sphaerospermum.*

Enzyme	System	K_m (M)	V_{max}
Invertase	Control	$2{\cdot}5 \times 10^{-5}$	0·91
	with NaCl	$7{\cdot}14 \times 10^{-3}$	2·5
Isocitrate lyase	Control	$1{\cdot}55 \times 10^{-5}$	38·4
	with NaCl	$1{\cdot}33 \times 10^{-4}$	333·0
Malate dehydrogenase	Control	5×10^{-5}	50·0
	with NaCl	$16{\cdot}6 \times 10^{-5}$	136·42

Sodium chloride (2M) was added *in vitro* to the assay mixture.

In order to investigate the effect of NaCl on the *de novo* synthesis of proteins *C. sphaerospermum* was grown in the normal medium for 24 h and transferred to medium containing sodium chloride and further incubated till 72 h in the presence and absence of cycloheximide. The activities of FDP aldolase, isocitrate lyase and malate dehydrogenase were found to increase in the absence of cycloheximide. But when a similar experiment was conducted in the presence of cycloheximide, there was no significant increase in the activity of isocitrate lyase suggesting that sodium chloride may be activating the *de novo* synthesis of this enzyme. Malate dehydrogenase and FDP aldolase however did not register similar effects under these conditions suggesting that sodium chloride may not be involved in activation of *de novo* synthesis of these enzymes. The activities of FDP aldolase and malate dehydrogenase were however found to be increased in the presence of cycloheximide, the reasons of which are not understood at present (table 3). This effect of cycloheximide in increasing the activities of some enzymes were shown earlier by us in *Neurospora crassa* (Shinde and Chhatpar, 1981).

At present we are not in a position to explain where and how much salt gets accumulated intracellularly. However, growing the fungus in the presence of different concentrations of salt was found to influence distinctly the activities of a number of enzymes.

Table 3. Isocitrate lyase, malate dehydrogenase and FDP aldolase activities from *C. sphaerospermum* grown in medium and transferred to medium in the presence of NaCl and with and without cycloheximide.

Growth conditions		Isocitrate lyase (units/mg protein)	Malate dehydrogenase (units/mg protein)	FDP aldolase (units/mg protein)
Initially grown on (for 24 h)	Transferred to (for further 48 h)			
Medium	No transfer	49·95	9·99	36·62
Medium + NaCl (1M)	No transfer	138·65	18·48	110·91
Medium	Medium + NaCl (1M)	255·14	15·00	131·32
Medium	Medium + NaCl (1M) + Cycloheximide	112·65	30·03	165·21

Several possible explanations can be given for these observations: (i) salt might have activated some enzymes post-translationally, (ii) salt might have increased the production of some enzymes by either increasing the rate of transcription or translation, (iii) both mechanisms might have been operating simultaneously for some enzymes, (iv) salt might have changed the turnover rates of enzymes. In our experiments sodium chloride was found to enhance the *de novo* synthesis of isocitrate lyase as well as activate the enzymes FDP aldolase, malate dehydrogenase, invertase and isocitrate lyase.

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