Isolation of Mitochondria from *Sarcophaga ruficornis* Larvae Rich in Phenolics

In connection with the investigation of respiratory enzymes in immature insects, an attempt was made to isolate mitochondria from *S. ruficornis* larvae. The homogenates prepared by conventional techniques turned brown and either no or very little activity could be detected. Apparently, the larval tissues contained the enzyme inhibitors. Enzymic browning is known to result from the polymerization of quinones formed from mono-or dihydric phenols by the action of phenol oxidase. Endogenous phenols are reported to inhibit several plant enzymes[1]. Since the phenol oxidase and phenols are invariably present in high concentrations in the cuticle and hemolymph of immature stages of insects[2,3], it seemed reasonable to attribute the browning of homogenates and subsequent enzyme inhibition in *S. ruficornis* larvae to interference by phenolics.

As far as the present authors are aware, no report is available to preclude phenolic interference during the isolation of mitochondria from larval stages of insects. The present communication describes the isolation and characterization of active mitochondria from *S. ruficornis* larvae by preventing the phenolic interference.

Materials and methods. The 3rd instar larvae of *S. ruficornis* were obtained from the running culture in the laboratory and their 20% homogenates prepared in A) 0.25 *M* sucrose, B) medium A plus 1% bovine serum albumin (BSA) and 0.01 *M* EDTA, C) medium B plus 0.005 *M* sodium metabisulphite and D) medium B plus 0.01 *M* freshly neutralized cysteine hydrochloride. Each homogenate was subjected to the following procedure. It was filtered through muslin and centrifuged at $150 \times g$ for 10 min to remove the debris. The supernatant was centrifuged at $3000 \times g$ for 10 min, the supernatant decanted and the residue washed with the grinding medium. The $3000 \times g$ supernatant was centrifuged at $8000 \times g$ for 10 min, the supernatant decanted and the residue washed with the grinding medium. The 3000 and $8000 \times g$ pellets were suspended in the grinding medium and used in the enzyme assay. All operations were carried out at 0–4 °C.

Cytochrome *c* oxidoreductase (cytochrome *c* oxidase) and succinate oxidoreductase (succinic dehydrogenase) were assayed according to COOPERSTEIN and LAZAROW[4] and SLATER and BONNER[5] respectively. The oxygen uptake in oxidative phosphorylation was measured according to SACKTOR and COCHARAN[6] using standard Warburg technique, and phosphorylation by the disappearance of orthophosphate from the reaction system by the method of FISKE and SUBBAROW[7]. The concentrations of BSA, EDTA, sodium metabisulphite and cysteine chosen favoured maximum activity of the enzymes studied.

Results. The results obtained are reported in the Table. The $3000 \times g$ sediment and $8000 \times g$ supernatant exhibited insignificant or no enzyme activity. Most of the activity was localized in $3000–8000 \times g$ pellet. The mitochondrial pellet isolated in 0.25 *M* sucrose was brown in colour and did not exhibit cytochrome *c* oxidase activity; the inclusion of BSA and EDTA restored only slight activity. Addition of sodium metabisulphite and cysteine to medium B caused respectively 2- and 5-fold increase in the cytochrome *c* oxidase activity.

Succinic dehydrogenase activity was very low when isolation medium A was used; the inclusion of EDTA and BSA caused only little enhancement. Supplementation of sodium metabisulphite and cysteine to medium B caused respectively 2- and 3-fold increase in the activity.

The P:O ratio for α-glycerophosphate oxidation was 0.51 when mitochondria were isolated in sucrose; it decreased to 0.45 on inclusion of EDTA and BSA. Addition of EDTA and BSA to the sucrose medium caused a two-fold increase in the P:O ratio for succinate oxidation. Incorporation of metabisulphite had little effect on the P:O ratios. Supplementation of cysteine to medium B, however, caused a marked increase of 2.6 and 4-fold in the P:O ratio, with α-glycerophosphate and succinate respectively. Succinate and α-glycerophosphate oxidations occurred at a relatively high rate with the mitochondrial preparation with medium A and B. However, phosphate esterification occured at a slow rate, so that the P:O ratios were very low. The use of cysteine was found to be superior to sodium metabisulphite.

Discussion. The medium for isolation of mitochondria from insect larval stages rich in phenolics requires careful formulation in order to recover maximum activity. The isolation of active mitochondria from plant tissues has been hampered in several cases by the presence of phenolic compounds[8]. Homogenates prepared in sucrose medium resulted in browning, probably due to the polymerization of quinones present in the cuticle, and either no or very little activity could be demonstrated. This may be due to the fact that quinones polymerize spontaneously and enter into comparatively stable irreversible covalent linkages with proteins[9]. Phenol oxidase is reported to be bound with the mitochondria of blow-fly[10]. Addition of BSA restored slight activity because the exogenous proteins are known to protect the enzyme by entering into hydrogen bonding and covalent linkages with phenols. BSA has been used extensively in the isolation of mitochondria from insect tissues[11], and in its absence oxidative phosphorylation could not be demonstrated in insect flight muscle mitochondria[12]. The use of EDTA in the

Enzyme activities and oxidative phosphorylation in *S. ruficornis*

Enzyme activity per g fresh weight[a]	Medium			
	A	B	C	D
Cytochrome *c* oxidase (mole/min)	—	0.06	0.12	0.316
Succinic dehydrogenase change in O.D./min	0.04	0.08	0.17	0.25
P:O ratio with glycerophosphate	0.51	0.45	0.65	1.33
P:O ratio with succinate	0.46	0.90	0.20	1.84

[a] The data presented is the mean of triplicate estimations.

[1] W. D. LOOMIS and J. BATTAILE, Phytochemistry *5*, 423 (1966).
[2] P. KARLSON and H. LIEBEU, Z. physiol. Chem. *326*, 135 (1961).
[3] R. H. HACKMAN, *The Physiology of Insects* (Ed. M. ROCKSTEIN; Academic Press, New York & London 1964), vol. 3.
[4] L. COOPERSTEIN and A. LAZAROW, J. biol. Chem. *189*, 665 (1951).
[5] C. E. SLATER and W. D. BONNER, Biochem. J. *52*, 185 (1959).
[6] B. SACKTOR and D. G. COCHARAN, Arch. Biochem. Biophys. *74*, 266 (1958).
[7] C. H. FISKE and Y. SUBBAROW, J. biol. Chem. *66*, 374 (1925).
[8] J. D. JONES and A. C. HULME, Nature, Lond. *191*, 370 (1961).
[9] K. H. GUSTAVSON, *The Chemistry of Tanning Process* (Academic Press, New York 1956).
[10] C. E. SERKERIS and D. MERGENHAGEN, Science *145*, 68 (1964).
[11] L. WOJTCRAZ and A. B. WOJTCRAZ, Biochim. biophys. Acta *31*, 297 (1959).
[12] P. S. KRISHNAN, P. N. VISWANATHAN and R. L. MATTOO, J. scient. ind. Res. *28*, 93 (1970).

homogenizing medium may be expected to inhibit the metal mediated enzymic or non-enzymic oxidation of phenols[12]. The cysteine tends to keep phenols in reduced condition[13]. The enhancement of enzyme activities by incorporating cysteine and sodium metabisulphite in the isolation medium was likely to be due to the fact that these reagents keep the phenols in their unoxidized non-inhibitory state.

The present communication reveals that enzyme isolation from immature stages of insects requires protection from phenols[14].

Zusammenfassung. Die Aktivitäten von Cytochrom-*c*-Oxydase und Succinat-Dehydrogenase sowie die oxydative Phosphorylation wurden in isolierten Mitochondrien von *S. ruficornis*-Larven durch Zusatz von BSA, EDTA und Cystein zu den Homogenaten vor Schädigung durch phenolische Substanzen geschützt.

G. N. VERMA and R. N. SHUKLA[15]

Department of Zoology, Lucknow University and Biochemistry Department, University of Lucknow, Lucknow 22 60 07 (India), 16 October 1974.

[13] J. W. ANDERSON and K. S. ROWAN, Phytochemistry *6*, 1047 (1967).

[14] Acknowledgment. The authors feel grateful to Prof. P. D. GUPTA for providing laboratory facilities and valuable help and to Prof. R. RAKSHPAL for encouragement.

[15] Biochemistry Department, University of Lucknow.

Action de leucine aminopeptidase I et II extracellulaires d'*Aspergillus oryzae* (I.P.410) sur des peptides synthétiques

Action of Leucine Aminopeptidase 1 and 2 (*Aspergillus oryzae* 410) on Various Synthetic Peptides

Au cours de travaux antérieurs, nous avons pu isoler et étudier dans un milieu de culture d'*Aspergillus oryzae* (I.P.410) 2 fractions extracellulaires à activité leucine aminopeptidasique (LAP)[1-4]. Comme une fraction extraite de *Serratia indica*[5], elles ne possèdent pas d'activité envers la leucine-amide mais possèdent une activité arylamidase envers la L-leucyl-4-nitroanilide (LpNa).

Nous avons pu observer que ces fractions étaient activables par la température et que la présence d'ions Mg^{++} augmentait l'activité de la fraction LAP 1 (50%). L'activité de LAP 2 par contre, ne subit aucune modification. L'oxydation du tryptophane de ces glycoprotéines par le N-bromosuccinimide amène une perte totale d'activité. La mise en évidence de liaisons *O*-glycosidiques par la méthode de «β élimination»[6] s'accompagne d'une perte totale de l'activité enzymatique et antigénique. Nous avons voulu ici comparer les activités de ces fractions vis à vis de peptides synthétiques[7].

Matériel et méthodes. La coupure de la liaison leucyl a été mise en évidence après incubation (24 h à 30 °C), en tampon phosphate de sodium (3 ml) 0,05 *M* pH 8, de 2,5 mg de dipeptide ou de 3,5 mg de tripeptide et de 500 µg de fraction enzymatique.

L'acide amine terminal libéré est identifié après chromatographie sur plaque de gel de silice dans un systeme *n*-butanol-acide acétique-eau (4.1.1) de 10 µl de milieu réactionnel. Il a été également dosé à partir de 10 µl de milieu d'incubation par le réactif ninhydrine-KCN à 570 nm.

Résultats et discussion. L'examen du Tableau nous permet de définir les différentes liaisons coupées par les fractions fongiques. Il apparait que la liaison leucyl est coupée de préférence par les 2 fractions, alors que ces dernières ne possèdent pas d'activité carboxypeptidase envers CBZ-alanine-leucine, ce qui justifie leur caractère leucine aminopeptidasique (LAP).

De manière identique à la fraction LAP 1 extraite également d'*Aspergillus oryzae* (460) par NAKADAI et al.[8] ces fractions n'ont aucune action sur les liaisons glycyl-glycine, gly-pro-ala ainsi que les liaisons gly-cyst-gly (glutathion). Une activité envers les liaisons glycyl, quand elle est associée à la leucine dans gly-leu-tyr semble exister dans le cas de LAP 1. On peut également noter une légère activité non spécifique envers des liaisons telles que tryptophanyl et tyrosyl et une activité, assez importante de LAP 1 sur les liaisons du type histidyl dans his-leu. Ces résultats sont confirmés par l'étude cinétique de la réaction en chromatographie sur plaque de gel de silice.

En outre, nous avons étudié l'activité de ces fractions sur le LpNa en présence d'alcools linéaires, à des concentrations croissantes compatibles avec leur miscibilité.

Action de LAP 1 et LAP 2 sur les différents peptides

	Δ D.O. à 570 nm	
	LAP 1	LAP 2
LEU-GLY-GLY	0,900*	0,900
GLY-PRO-ALA	0	0
GLY-LEU-TYR	0,600	0,100
GLY-CYS-TYR (Glutathion)	0	0
TRY-TYR	0,250	0,300
TRY-ALA	0,150	0
TYR-GLY	0,300	0
LEU-GLY	0,900	0,600
HIS-LEU	0,600	0,250
TYR-PHE	0,100	0,230
GLY-GLY	0	0
CBZ-ALA-LEU	0	0

* L'activité maximale étant déjà atteinte après 4 h d'incubation. Les résultats sont donnés en différence de densité optique à 570 nm (dosage à la ninhydrine) des milieux réactionnels avant et après incubation.

[1] J. P. LABBÉ et P. REBEYROTTE, Biochemie *56*, 839 (1974).

[2] P. REBEYROTTE et J. P. LABBÉ, C.r. Acad. Sci., Paris *274*, 2370 (1972).

[3] J. P. LABBÉ, P. REBEYROTTE et M. TURPIN, C.r. Acad. Sci., Paris *278*, 2699 (1974).

[4] J. P. LABBÉ, P. REBEYROTTE, E. MOREL et M. TURPIN, C.r. Acad. Sci. Paris *279*, 2139 (1974).

[5] A. TJEDER et B. HOFSTEN, Acta chem. scand. *217*, 1721 (1967).

[6] K. TANAKA et W. PIGMAN, J. biol. Chem. *240*, 1487 (1965).

[7] J. P. LABBÉ, en préparation.

[8] T. NAKADAI, S. NASUNO et N. IGUCHI, Agric. biol. Chem. *37*, 757 (1973).