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UV-irradiation or high incubation temperature increases the NAD-incorporation by mungbean seedlings

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Abstract. When mungbean seedlings were grown at 20° to 30°C, NAD-incorporation (ADP-ribosylation) activity of the crude chromatin preparation increased as temperature increased. NAD was incorporated into various chromatin protein fractions. When total counts per minute (cpm) was used for comparing the incorporation by the different protein fractions, then the 0.14 M NaCl extract was the highest, the histone fraction the second highest followed by the hot SDS-soluble protein fraction and the phenol-soluble protein fraction. When the incorporation comparison was based on cpm per mg protein, then the phenol-soluble protein fraction was the highest, the 0.14 M NaCl extract the second highest and histone fraction the 3rd highest followed by the hot SDS-soluble protein fraction. UV irradiation on mungbean seedlings caused increased *in vitro* NAD-incorporation activity. The longer the total irradiation time during the discontinuous irradiation, the higher the activity. With a constant total irradiation time, the longer the individual irradiation periods, the higher the activity.

Key words: Chromatin proteins; *Phaseolus radiatus*; Poly-ADP-ribosylation.

Introduction

Control of cellular reactions is often carried out by the covalent modification of previously synthesized enzymes, receptors, structural proteins etc. The covalent ADP-ribosylation of proteins is one such regulatory mechanism.

ADP-ribosylation may be either monomeric or polymeric. Mono-ADP-ribosyl transferases include an array of bacterial toxins and a number of endogenous cellular enzymes (Althaus and Richter, 1987). Poly-ADP-ribosylation reactions occur on chromatin proteins (Althaus and Richter, 1987). Poly-ADP-ribosylation reactions are also an integral part of the cellular response to DNA-damaging agents (Durkacz *et al.*, 1980; Skidmore *et al.*, 1979).

Although the occurrence of poly (ADP-ribose) polymerase in tissues of higher plants has been reported (Bocher and Szopa, 1982; Lin, 1976; Payne and Bal, 1976; Whitby *et al.*, 1979) little is known about its physiological functions. Increased poly (ADP-ribose) polymerase activity of mungbean seedlings at elevated incubation temperature and after UV-irradiation is reported in this work.

Materials and Methods

Plant Material

Seeds of mungbean (*Phaseolus radiatus*) were obtained from Asian Vegetable Research and Development Center (AVRDC), Tainan, Taiwan.

Germination Procedure

Seeds of *Phaseolus radiatus* were soaked in 0.1 - 0.5% H_2O_2 for 20 min and then in distilled water overnight. The imbibed seeds were placed on petri dishes with wet cheese cloth and then transferred to growth incubators set at 20°, 25°, or 30°±0.5°C in dark for 7 days. The cheesecloth was kept wet during germination.

Table 1. *UV irradiation procedure applied to mungbean seedlings*

Treatment	Germination Time		
	3rd day	4th day	5th day
Control	No treatment	No treatment	Harvesting
1	No treatment	UV 15 min→ 3 h in dark → UV 15 min→ 3 h in dark → UV 15 min → in dark.	Harvesting
2	UV 15 min → 3 h in dark → UV 15 min → 3 h in dark → UV 15 min → in dark.	Same as the 3rd day.	Harvesting
3	No treatment	UV 30 min → 3 h in dark → UV 30 min → 3 h in dark → UV 30 min → in dark.	Harvesting

UV-irradiation Procedure

Seeds of *Phaseolus radiatus* were germinated at 20°±0.5°C as described above with or without UV-irradiation. The UV-irradiation treatments that were applied are detailed in Table 1. In treatment 1, the seedlings were subjected to UV irradiation on the 4th day only. It consisted of three 15 min irradiations that were separated from one another by 3 hr of darkness. The seedlings were harvested on the 5th day. In treatment 2, the same UV treatment as is described for treatment 1 was used but it was applied on the 3rd as well as the 4th days. In treatment 3, the experimental conditions were the same as for treatment 1 except that the individual UV-irradiation periods were extended from 15 to 30 min. Seedlings were placed 20 cm below a Mineralight (a product of Ultra-Violet Prod. Inc., San Gabriel, CA, USA; 115 volts, 0.6 amp) which provided an irradiated area of 7 × 15.5 cm^2 at this height.

Preparation of Crude Chromatin

This was done mainly according to Huang and Bonner (1965). About 10-100 g of fresh etiolated seedlings was homogenized at 4°C with a fruit blendor in 5 vol (v/w) of grinding medium consisting of 0.25 M sucrose, 0.05 M Tris-HCl pH 8.0, and 0.001 M $MgCl_2$. The homogenate was filtered successively first through a single layer, then two layers and finally four layers of cheesecloth. The filtrate was next centrifuged at 4300 x g for 30 min. Crude chromatin was scraped from the surface of the pellets and then suspended in distilled water with a Potter-Elvehjem hand homogenizer. For immediate use, the homogenized chromatin was dialyzed twice against 100 volumes of distilled water for 2 hr and used as an enzyme source after storage for 2 days (crude chromatin). The dialyzed crude chromatin was subjected to further partial purification when needed.

Partial Purification of Chromatin

All operations were carried out at 4°C. The dialyzed crude chromatin was layered on top of about 40 ml of grinding medium described above except that 0.25 M sucrose was replaced by 1.78 M sucrose. Centrifugation was carried out at 12,000 x g for 30 min. The layer floating on top after centrifugation was discarded while the underlying layer was collected, dialyzed as described above and then stored for 2 days before use.

Determination of Protein

The protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Assay of NAD-Incorporation

The reaction mixture contained 40 mM Tris-HCl, pH 7.4, 10 mM MgCl, 5-10 μl of ^{14}C-(adenine-U)-NAD (yielding 20,000-60,000 cpm) together with 15-20 μl of 2.5 mM unlabelled NAD and crude chromatin or partially purified chromatin containing 400 μg or less protein in a final volume of 250 μl. The reaction was carried out at 25°C for 15 min. Chromatin preparation pre-

heated at 80°C for 10 min was used as a control. At the end of the reaction, the mixtures were treated in two different ways—one for total incorporation and the other for incorporation into various chromatin fractions.

For the determination of the total NAD-incorporation, each reaction mixture was combined with an equal volume of 20% (w/v) trichloroacetic acid (TCA) to stop enzyme activity. Then 50 μl of each reaction mixture was pipetted onto separate Whatman #1 filter paper disks, and the disks were batch washed in cold 5% TCA which contained 10% $Na_4P_2O_7$, cold 5% TCA, 95% ethanol, and finally ether (Yoneda and Bollum, 1965). Radioactivity of the dried disks was counted in 0.4% PPO (w/v) and 0.024% (w/v) POPOP in toluene in a Beckman liquid scintillation counter LS-100C. All data after correction from control tests were calibrated using a standard curve with counting efficiency versus amounts of proteins in reaction mixtures usually in the range of 2000 to 40000 cpm.

For determination of NAD-incorporation into various chromatin proteins, the reaction mixtures were each combined with an equal volume of 20% TCA and centrifuged at 30,000 x g for 20 min at 4°C. The pellets were washed 3 times by the same centrifugation procedure each time with ca. 100 vol of ice-cold 50 mM Tris-HCl, pH 7.4. The various chromatin proteins were then extracted from the washed material as described below and the amounts of incorporated radioactive NAD were determined as described above. The washed chromatin containing radioactivity was extracted by homogenizing with 100 vol of 0.14 M NaCl in 50 mM Tris-HCl, pH 7.4 (Wang, 1968) and centrifuged as mentioned above. The supernatant was "non-histone protein extract". The pellets were then extracted with 40 vol of 0.25 N HCl (Le Stourgeon and Rusch, 1973) and centrifuged as mentioned above. The supernatant was "histone fraction". The remaining pellets were extracted with 20 vol of chloroform-methanol containing 0.25 N HCl and centrifuged as mentioned above. The supernatant which contained lipids and phospholipids was discarded. The pellets were further extracted with 20 vol of ether and centrifuged again. The supernatant was also discarded. The pellets were then extracted with phenol according to the method of Teng *et al.* (1970) and the supernatant was called "phenol soluble proteins". The residual chromatin pellets were then washed 3 times with 50 vol of 10% methanol to remove the remaining phenol and resuspended in 20 vol of 0.01 M phosphate buffer (pH 7.2) containing 0.14 M 2-mercaptoethanol and 5% sodium dodecyl sulfate (SDS). This preparation was heated in boiling water for 3 min to solubilize the remaining proteins. The proteins thus solubilized constituted "the hot SDS-soluble protein fraction".

Chemicals

^{14}C-(adenine-U)-NAD (271 mCi/m mole) was purchased from The Radiochemical Center, Amersham, Bucks, U.K.; Tris (hydroxymethyl) aminomethane, oxidized form of nicotinamide adenine dinucleotide (β-NAD) and bovine serum albumin were products of Sigma Chemical Co., St. Louis, Mo. U.S.A.; Folin Ciocalteu reagent was purchased from Wako Pure Chemical Ltd., Osaka, Japan; Trichloroacetic acid, 2,5-diphenyloxazole (ppo) and 1,4-bis-(2-(5-phenyloxazolyl))-benzene (popop) were obtained from E. Merck, Darmstad, Germany.

Results

Effect of Germinating Temperature on NAD-incorporation Activity

Table 2 shows that NAD-incorporation activity of mungbean seedlings of three varieties increased as incubation temperature increased within the tested range of 20° to 30°C. When the logarithm of the NAD-incorporation activity was plotted against temperature, near straight lines were obtained (data not shown).

Table 2. *Effect of germinating temperature on NAD-incorporation activity*

Temperature	NAD-incorporation (pmol/mg protein)			
	Variety 1	Variety 2		Variety 3
		Exp. 1	Exp. 2	
20°C	712	412	198	1090
25°C	1340	956	900	3490
30°C	4250	1920	2230	6730

Effect of UV Irradiation on NAD-incorporation Activity

Table 3 shows that UV irradiation caused increased NAD-incorporation activity (treatment 1, 2, or 3 versus control). The longer the total irradiation time during the discontinuous irradiation the higher the activity (treatment 2 or 3 versus treatment 1). With a constant total irradiation time, the longer the individual irradiation periods, the higher the activity (treatment 3 versus 2).

NAD-incorporation into Various Chromatin Proteins

Table 4 shows the incorporation of ^{14}C-(adenosine-U)-NAD into various 5% TCA-insoluble protein fractions of chromatin. When total counts per minute (cpm) was compared, the 0.14 M NaCl extract was the highest, the histone fraction the second highest followed by the hot SDS-soluble protein fraction and the phenol-soluble protein fraction. When the cpm per mg protein was compared, the phenol-soluble protein fraction was the highest, the 0.14 M NaCl extract the second highest, and the histone fraction the 3rd followed by the hot SDS-soluble protein fraction.

Table 3. *Effect of UV irradiation at a germination temperature of 20°C on the rate of NAD-incorporation into mungbean chromatin*

Treatment	NAD-incorporation (pmol/mg protein)			
	Variety 4		Variety 5	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Control	74	73	73	10
1	95	92	90	13
2	380	375	372	47
3	741	733	729	71

Discussion

For unknown reasons total incorporation or specific activity of incorporation of NAD varied to some extent when data of comparable sets of experiments using different batches of mungbean or different chromatin preparations of the same batch of mungbean were examined (Lin, 1984). The same phenomena were observed again in this work. However the variation does not affect the general conclusions drawn here.

ADP-ribosylation of proteins is a rapidly expanding area of research. The number of publications in this field has been growing exponentially since 1980 (Althaus and Richter, 1987). ADP-ribosylation is involved in the autonomous regulation of adenylate cyclase (Jacquemin *et al*., 1986), and in the induction of B-cell differentiation antigens in Daudi cells (Exley *et al*., 1987). It may also signal changing metabolic conditions to the chromatin of mammalian cells (Loetscher *et al*., 1987). Reversible ADP-ribosylation has been demonstrated to be a regulatory mechanism in prokaryotes by heterologous expression (Fu *et al*., 1990). Guanine nucleotide-binding proteins that enhance choleragen ADP-ribosyltransferase activity were found recently (Price *et al*., 1988) and at least one ADP-ribosylation factor is functionally and physically associated with the Golgi complex (Stearns *et al*., 1990).

We have provided lines of evidence showing the occurrence of mono- and poly-(ADP-ribose) synthesizing enzyme(s) via NAD-incorporation in crude chromatin isolated from germinating seedlings of *Phaseolus radiatus* and *Oryza sativa* (Lin and Chang, 1980; Lin and Kuo, 1981).

The most impressive finding in this work is the effect of germinating temperature on the NAD-incorporation activity. Within the tested range, increasing

Table 4. *Incorporation of ^{14}C-(adenosine-U)-NAD into various 5% TCA-insoluble protein fractions of chromatin obtained from mungbeans germinated at 25°C*

Fraction	Total counts (cpm)	% of total counts	cpm per mg protein
0.14 M NaCl extract	1.0×10^5	73.3	1.0×10^4
Histones	2.9×10^4	21.2	4.7×10^3
Phenol-soluble proteins	3.5×10^3	2.6	2.4×10^4
Hot SDS-soluble proteins	4.0×10^3	2.9	2.4×10^3

germinating temperature raises the enzyme activity almost exponentially similar to the effect of 2,4-D on the enzyme activity (Lin, 1984). It was reported that DNA synthesis in growing tissues, i.e. chick-embryo -liver nuclei, containing polyamines at high levels, such as is the case with tumors and the fetus, is stimulated by polyamine-mediated ADP-ribosylation of the nuclear proteins (Tanigawa *et al.*, 1980). Whether this is true also in mungbean seedlings deserves further study.

Among various physiological functions suggested for ADP-ribose in animal cells, one is to participate in the cellular recovery from DNA damage (Boulikas, 1989; Durkacz *et al.*, 1980; Schraufstatter *et al.*, 1986; Skidmore *et al.*, 1979; Yamamoto *et al.*, 1981). We demonstrate here that ADP-ribosylation activity in crude chromatin is enhanced after UV irradiation of mungbean seedlings (Table 3). Because the data of temperature treatment are much more consistent than that of UV-irradiation (cf. Tables 2 and 3), there is at least one possible explanation for data variation of the comparable sets of experiment in this work: the technical difficulties involved in exposing seedlings uniformly to UV radiation.

The order of NAD-incorporation into various protein fractions of chromatin of mungbean seedlings (Table 4) is quite different from that of rat liver chromatin (Lin, 1975) no matter whether total incorporation or specific incorporation is compared. The fraction referred to as the 0.14 M NaCl extract, had the highest total incorporated cpm, the histone fraction the 2nd highest, followed by the hot SDS-soluble protein and the phenol-soluble protein fractions; on the other hand, the phenol-soluble protein fraction had the highest specific incorporation (cpm per mg protein), the 0.14 M NaCl extract the 2nd highest, and the histone fraction the 3rd followed by the hot SDS-soluble protein fraction when dealing with the mungbean seedling chromatin. In rat liver chromatin, the histone fraction is the highest, the phenol-soluble protein fraction the 2nd highest, the 0.14 M NaCl extract the 3rd and this is followed by the hot SDS-soluble protein fraction no matter whether the results are based on total or specific incorporation.

That the 0.14 M NaCl extract of mungbean seedling chromatin has the highest total incorporation is a new and significant finding. Proteins of this fraction, usually called non-histone proteins or acidic proteins, are considered to play regulatory functions in contrast to histones (except Hl) which are essential parts of chromatin structure. In chick-embryo-liver nuclei, the major ADP-ribosylated proteins were determined as two non-histone proteins of M_r 130,000 and 70,000 (Tanigawa *et al.*, 1980). Some published evidence indicates that perhaps poly-(ADP-ribose) polymerase (indirectly) modulates the activity of DNA ligase ll (Althaus and Richter, 1987). Questions such as: Do ADP -ribosylation of the above mentioned proteins occur in higher plant tissues such as mungbean or rice seedlings?; How many other nonhistone proteins are ADP -ribosylated and what are these?; are urgent topics to be studied in order to understand the phsiological functions of ADP-ribosylation in higher plants.

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紫外綫照射或高溫發芽增加綠豆芽之 NAD 聚合活性

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當綠豆在攝氏 20 至 30 度之間發芽時，其染色質抽取液之 NAD-incorporation(亦即 ADP-ribosylation)活性隨著溫度升高而增加。NAD 被聚合進入染色質之不同蛋白質部分：當以放射性(cpm)總量爲比較基準時，0.14 M氯化鈉之抽取部份排名第一，而 histone 部份排名第二，接著爲熱 SDS-可溶蛋白質部份及酚可溶蛋白質部份；但以每毫克之 cpm 爲準時，酚可溶部份高居第一，0.14 M 氯化鈉之抽取部份排第二，histone 部份第三，接著爲熱 SDS-可溶部份。紫外綫照射增加染色質抽取液的 NAD 聚合活性。在紫外綫照射與黑暗交替之不連續情況下，累積的照射時間越長(即每次個別照射時間相同而照射次數多者；或照射次數相同而每次個別照射時間長者)其活性越高；而當累積的紫外綫照射時間相同但照射次數不同時，每一個別照射時間越長者其活性越高。