IN VITRO SYNTHESIS OF A SPECIFIC PROTEIN VIZ. ASPARAGINE SYNTHETASE BY NUCLEAR FRACTION FROM GAMMA IRRADIATED POTATO BUDS

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Summary: Irradiation induced synthesis of asparagine synthetase was localized in the nucleus of the potato buds. Isolated nuclei were capable of incorporating amino acids into asparagine synthetase protein and the process was inhibited by cycloheximide (100%), puromycin (73%) and by feed back inhibitor β -asparyl hydroxamate (100%). Preincubation of the bud tissue with actinomycin D was necessary to express its inhibitory effect.

Introduction

The nuclear protein synthesis has an important biological role. Cell nuclei are capable of protein synthesis, which is denoted by the in vitro labelling of protein with isolated nuclei occurring within the nuclear domain (1). Isolated nuclei are usually used in these studies in order to overcome the difficulties encountered in using intact cell, where there is transport of protein across the membrane. Although many workers (2, 3, 4) have demonstrated the synthesis of protein by isolated nuclei by studying the incorporation of ^{14}C-amino acids into nuclear protein, still, an unequivocal proof for nuclear protein synthesis is lacking. When nuclear protein synthesis is studied, it is best to use a protein which is not synthesised in extra nuclear domain. Also it is better that this protein has a definite biological function and can be well characterized by purification. Till now no such protein is known to

be synthesised by the nuclei. The present investigation describes the synthesis of an enzyme protein viz. asparagine synthetase, localized in the nuclei from gamma irradiated potatoes.

Experimental

Isolation of nuclei

Buds were excised from irradiated potatoes and incubated for 4½ hrs in distilled water, which is the time taken for maximum synthesis of asparagine synthetase (5). The nuclei was isolated according to the procedure of Birnstiel et al (6) slightly modified to suit our purpose. Buds were then rinsed with water and placed in a cold mortar containing equal volume (100 buds - 100 ml) of grinding medium (0.4 M sucrose, 0.002 M $CaCl_2$ and 10^{-3} M). The buds were pressed with pestle and ground for 1 min. The ground mass was passed through 4 layers of cheese cloth by squeezing well by rubbing between thumb and index fingers. The filtrate was centrifuged at 750 g for 10 min. The supernatant was kept for activity determination. The residue was suspended again in grinding medium and centrifuged at 600 rpm (50 g) for 30 sec to remove starch particles. The supernatant was again centrifuged at 750 g for 10 min. The nuclear pellet was washed once more in grinding medium and suspended in about 4 ml of this medium. Microscopic examination after staining with aceto-carmine revealed about 90% whole nuclei in this preparation without any cytoplasmic contamination. 0.3 ml of this suspension was used for the assay of asparagine synthetase activity by estimating the hydroxamate formed (5).

In vitro synthesis of asparagine synthetase

In these experiments nuclear fraction was isolated immediately after irradiation. The whole operation took about 50 to

Table I

Localization of asparagine synthetase

Fraction	Specific Activity Hydroxamate formed n moles/mg protein
Homogenate	31.2
Nuclear fraction	148.0
Supernatant	11.3

60 min after irradiation. The nuclear fraction thus obtained was incubated at 37° for 4½ hr with and without amino acid incorporating system. The reaction mixture consisted of 2 ml 0.05 M Tris-HCl buffer containing 0.4 M sucrose and 0.002 M $CaCl_2$, pH 7.2, 0.5 mM $MgCl_2$; 1 mM KCl; 100 µM ATP; 20 µM GTP; mixture of 18 amino acids 50 µM each; 100 µM DL-leucine 1-^{14}C (47.9 mCi/mmole) 20 µCi; 1.5 ml nuclear suspension to a final volume of 4 ml. After incubation the reaction mixture was centrifuged and nuclear pellet was suspended in 1.5 ml grinding medium 0.3 ml aliquot was used for asparagine synthetase assay. The radioactivity incorporated into protein was determined after precipitating the protein with 20% TCA and washing several times with 5% TCA, distilled water and finally alcohol. The residue was dissolved in 0.1 N NaOH. Protein was estimated using biuret method (7).

Results and Discussion

The bud tissue from irradiated potatoes were capable of de novo synthesis of asparagine synthetase, which was a fast reaction

Table II

Efficiency of nuclear fraction from irradiated potatoes for *in vitro* synthesis of asparagine synthetase

Source of nuclear fraction	Asparagine synthetase specific activity n moles/mg protein		^{14}C-leucine incorporation cpm/mg protein	
	Control	With complete system	Control	With complete system
Unirradiated	Nil	Nil	1225	2872
Irradiated	32	125	1.6×10^5	4×10^5

Experimental details are described under 'experimental'. 'Control' denotes that amino acid incorporating system is omitted in the incubation mixture.

initiated within 3 hrs after irradiation and reached a maximum in 5 hrs (5). By this time the synthesis ceased and there was turnover of this protein, which was associated with the synthesis of another protein. The transient formation of this enzyme seemed to have some definite function. Therefore, it was important to know the site of its synthesis. For this purpose the bud tissue was homogenised after allowing maximum synthesis of asparagine synthetase. The nuclear and cytoplasmic fractions were tested for asparagine synthetase activity (Table I). Almost all activity was present in the nuclear fraction suggestive of its synthesis in nucleus. This finding led us to check the activity of nuclei to label the nuclear proteins *in vitro* and particularly for the synthesis of asparagine synthetase assessed by its activity. The capability of nuclear fraction for *in vivo* and *in vitro* incorporation of labelled amino acids is influenced by the metabolic activity of the tissue. Our

Table III

Effect of inhibitors on the synthesis of asparagine synthetase

Inhibitors		Asparagine synthetase specific activity n moles/mg protein	^{14}C-leucine incorporation cpm/mg protein
None		150	5×10^5
Puromycin	25 µg/ml	41	1.2×10^5
Cycloheximide	10 µg/min	0	1.5×10^5
Actinomycin D	10 µg/ml	130	3.8×10^5
Actinomycin D[a]	10 µg/ml	0	2000
β-aspartyl hydroxamate	10^{-4} M	0	9.7×10^4

The inhibitors were added into the reaction mixture containing amino acid incorporating system at concentration mentioned above. Actinomycin D[a] indicates that buds were preincubated with actinomycin D for ½ hr before the isolation of nuclei.

earlier results clearly indicated that there was a transient activation of the metabolic activity (8, 9) of potatoes after gamma irradiation. Nuclear fraction isolated from unirradiated potatoes was neither capable of incorporating radioactivity into their proteins nor of showing any asparagine synthetase activity. Nuclear fraction from irradiated tuber was capable of synthesising protein very efficiently, as shown by the increase in ^{14}C-leucine incorporation into soluble protein, and also could synthesise asparagine synthetase (Table II). Supplementation of the amino acid incorporating system in the reaction mixture resulted in an increased incorporation as well as enzyme activity.

The sensitivity of this amino acid incorporation into protein and the development of asparagine synthetase activity towards protein synthesis inhibitors and actinomycin D was next investigated (Table III). Cycloheximide at 10 µg/ml level could inhibit the formation of asparagine synthetase completely while amino acid incorporation was affected only 70%. Puromycin was less effective. It could bring about 73% inhibition of asparagine synthetase formation. The specific and potent feed back inhibitor for 'asparagine synthetase' synthesis viz. β-aspartyl hydroxamate (5) was very effective inhibitor both for ^{14}C-incorporation (80%) and asparagine synthetase formation (100%). Results on inhibition studies with actinomycin D implicated the necessity for RNA synthesis prior to the synthesis of asparagine synthetase. When nuclear fraction was isolated and incubated with actinomycin D (10 µg/ml) no effect was discerned. On the other hand preincubation of buds for ½ hr with actinomycin D before the isolation of nuclear fraction was highly effective in controlling both incorporation and enzyme activity. So it seemed most probable that within the time taken to isolate the nuclei from the buds after irradiation, the RNAs required for the synthesis of protein were already made. This observation fitted in well with our earlier results (9) that the capacity for maximum RNA synthesis lasts only for about ½ hr in irradiated buds. These studies have demonstrated an energy dependent incorporation of L-^{14}C-amino acids into a specific protein and this process was entirely dependent upon the synthesis of a discrete RNA molecule.

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