Changes in Fructose-2,6-bisphosphate Level during the Growth of Suspension Cultured Cells of *Catharanthus roseus**

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The level of fructose-2,6-bisphosphate (F2,6BP), a potent regulator of carbohydrate metabolism, varied from 23 to 448 pmol·g fresh weight^{-1} during the growth of the cells of *Catharanthus roseus* in suspension culture. The maximum F2,6BP level was found in the cells at the lag phase of the growth, and its level decreased with cell division and enlargement.

Introduction

Fructose-2,6-bisphosphate (F2,6BP) was discovered as a potent stimulator of phosphofructokinase by van Schaftingen *et al.* [1]. Other findings indicate that F2,6BP also modulates the activity of some plant enzymes, including pyrophosphate dependent phosphofructokinase (PPi-PFKase) and fructose-1,6-bisphosphatase [2].

Since F2,6BP was demonstrated in mung bean seedlings [3], the levels of F2,6BP have recently been determined in some plant materials [3–8]. These data indicate that the F2,6BP level fluctuates rapidly, with the accompanyment of several physiological phenomena.

As a part of our study on metabolic regulation in cultured plant cells, changes in the F2,6BP level during the growth of *Catharanthus roseus* in batch suspension culture were determined. This is the first report of F2,6BP levels being determined in cultured plant cells.

Materials and Methods

Cells from a batch suspension culture of *Catharanthus roseus* grown in Murashige-Skoog medium were used as the material for this work. The culture conditions were essentially the same as we described in our previous papers [9–11].

Sulphate free preparations of PPi-PFKase (from potato tubers), aldolase, glycerophosphate dehydrogenase and triosephosphate isomerase were obtained from Sigma Chemical Company, St. Louis, and these enzymes, which were supplied as lyophilized powder, were dissolved in 50% glycerol and stored at −20 °C.

Extraction and assay of F2,6BP were performed using the methods described by van Schaftingen [12]. The homogenization of the cells (500 mg fresh weight) was carried out using a Potter-Elvehjem type homogenizer with 5 ml of 0.1 M KOH. The pH of the resulting homogenate should be above 11 to denature proteins and stabilize F2,6BP. The degree of cell disruption was checked under a microscope. More than 90% of the cells were broken during this treatment. The homogenate was heated at 80 °C for 5 min and then cooled in an ice bath. It was centrifuged at $20000 \times g$ for 20 min at 2 °C, and the supernatant was used directly as the plant extract for the F2,6BP assay.

The assay method is based on the activation of potato tuber PPi-PFKase by F2,6BP [12]. A spectrophotometric assay of PPi-PFKase is employed based on the coupling of fructose-1,6-bisphosphate formation to the oxidation of NADH with the use of sequential reactions catalyzed by aldolase, triosephosphate isomerase and glycerophosphate dehydrogenase [14]. The assay mixture contained 50 mM Tris-acetate buffer (pH 8.0), 2 mM magnesium acetate, 2.5 mM fructose-6-phosphate, 17.5 mM glucose-6-phosphate, 0.2 mM NADH, 0.5 mM tetrasodium pyrophosphate, approx. 0.1 mU PPi-PFKase, 1 U aldolase, 10 U triosephosphate isomerase, 1 U glycerophosphate dehydrogenase

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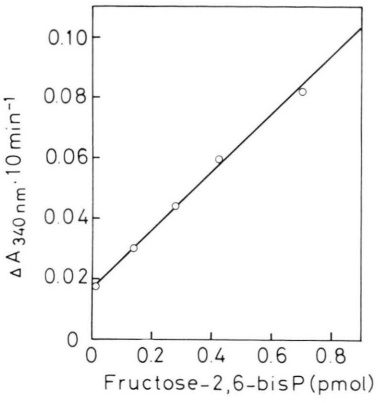

Fig. 1. A typical standard curve for the assay of fructose-2,6-bisphosphate.

and 10–40 µl plant extract in the total volume of 1 ml.

In order to remove F2,6BP as a contaminant in fructose-6-phosphate and glucose-6-phosphate, solutions of these compounds were treated with HCl and then neutralized as described in ref [12]. The exact concentration of standard F2,6BP in the stock solution was determined as the amount of fructose-6-phosphate appearing in the course of a mild acid treatment.

After the plant extract had been incubated for 5 min with the assay mixture mentioned above without PPi, PPi was added to the mixture and the decrease of absorbance at 340 nm was measured using a Hitachi double beam spectrophotometer type U-3200, which was fitted with an accessory for enzymatic analysis.

A linear relationship between the amount of F2,6BP and absorbance was obtained up to 2 pmol of F2,6BP (Fig. 1 and unpublished data). For determination of the recovery of F2,6BP concentration, known amounts of F2,6BP were always added to a part of the plant extract before the assay and the value obtained was adjusted. The recoveries were 74–104%. In the preliminary experiments, known amount of F2,6BP was added to the homogenate before heating. Similar recovery of added F2,6BP was obtained.

Results and Discussion

In our series of studies using cultured *Catharanthus roseus* cells, we have measured several metabolites and enzyme activities during the growth of the cells in suspension culture [9, 11, 13]. F2,6BP, an important signal of carbohydrate metabolism, has recently been discovered [1, 7], and its presence in plant cells has been reported [3–8]. We have, therefore, determined its level in the cultured cells in order to elucidate the regulatory mechanism of metabolic change in the cells during growth. As shown in the Materials and Methods section, the assay system of the compound in *Catharanthus* cells was established by modifying the method used by Van Schaftingen [12].

Changes in F2,6BP level during the growth of cultured *Catharanthus roseus* cells are shown in Fig. 2. The level varied from 23 to 448 pmol·g fresh weight^{-1}. It has been suggested that F2,6BP was localized in the cytosol of plant cells [4, 5]. If the cytoplasm comprises 5% of the volume of cultured plant cells, as estimated by Meyer and Wagner [15], cytoplasmic concentration of F2,6BP is calculated to be 0.46–8.96 µM. The values obtained here are not very different from the values for some other non-photosynthetic plant tissues [6, 8]. In dormant Jerusalem artichoke tubers, the level of F2,6BP was 10–150 pmol·g fresh weight^{-1}, and it increased during the dormancy break and reached 2.5–5.0 nmol·g fresh weight^{-1} [6]. The F2,6BP level in castor bean

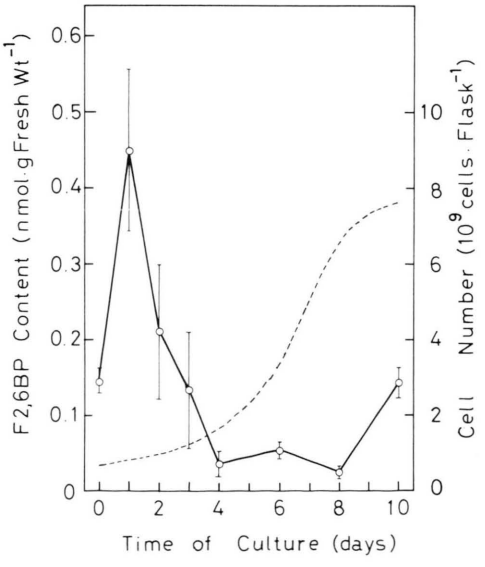

Fig. 2. Changes in fructose-2,6-bisphosphate level during the growth of the suspension cultured cells of *Catharanthus roseus*. The data points represent the mean ± SE of more than 4 experiments. Dotted line shows the mean cell number per flask as reference.

endosperm is 185 pmol·endosperm^{-1} (equivalent to 213 pmol·g fresh weight^{-1}) at the 4th day of germination [8].

F2,6BP is a potent activator of PPi-PFKase and inhibitor of fructose-1,6-bisphosphatase in plant cells [2]. The K_a value for plant PPi-PFKase is usually 10 nm−3 μM. This is dependent both on assay conditions and the source of enzymes [2, 6, 14]. The level of F2,6BP found in the *Catharanthus* cells seems to be at or slightly below the concentration required for the full activation of PPi-PFKase in the cells.

The F2,6BP level rose sharply on the 1st day, and then gradually decreased. The level increased again slightly at the end of the culture period (Fig. 2). Kanamori *et al.* reported that respiration, *i.e.* O_2 uptake in the *Catharanthus* cells increased during the early phase of cell growth [9]. Although there is evidence of a preferential contribution of the oxidative pentose phosphate pathway to the carbohydrate oxidation in the lag phase of the cells, activity of the Embden-Meyerhof-Parnas pathway also increases during the early phase of cell growth [9]. The high F2,6BP level may activate PPi-PFKase which is now recognized as one of the enzymes of the latter pathway in higher plants.

Formation of F2,6BP is catalyzed by 6-phosphofructo-2-kinase. The presence of this enzyme has been demonstrated in higher plants as well as other organisms [6]. The level of the substrates of the enzyme, fructose 6-phosphate and ATP, also increased rapidly when the stationary phase cells were transfered to the new medium [16] and [Ashihara (unpublished results)]. Therefore, the increase in F2,6BP level in the cells may be partly due to the increase in the substrates.

However, detailed studies are necessary before the actual function of F2,6BP and the regulation of its level in plant cells can be fully elucidated.

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