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Variations of adenyl nucleotide content in two potato cultivars stored at 15 °C. I. Method for the determination of ATP level and first results

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Summary

A method for determining ATP content based on sulfuric acid extraction and luciferin-luciferase bioluminescence assay was developed for potato tubers. The inactivation of apyrase by a thermal treatment as well as the ATP stability in acidic potato extracts stored at -25 °C have been particularly considered. The proposed method is simple, reliable, sensitive and can be used for automatic purposes. This method has been applied to the study of the variation of ATP levels in stored potato tubers (cvs Ukama and Granola). The large changes occurring over a 9 month period are discussed in correlation with dormancy, sprouting and tuberization.

Introduction

The post-harvest physiology of potato tubers has been extensively studied for fundamental, agronomical and economical reasons. The physiological and biochemical state of seed potato tubers at the end of storage is essential because it greatly influences the yield of the future crop. Moreover, the quality of ware potatoes used for consumption and industrial processing depends also on these parameters.

During storage, potato tubers are characterized by two major physiological periods, the dormancy and the incubation. The dormancy period characterizes the state of the tuber until sprouting which is followed by the incubation period, at the end of which tuberization occurs. Each of these physiological periods is accompanied by changes in the content of several biochemical compounds, among which carbohydrates (Burton, 1965; Rumpf, 1972; Reust & Aerny, 1985; Linnemann et al., 1985; Van Es & Hartmans, 1986; Richardson et al., 1990; Spychala & Desborough, 1990), proteins (Mulder & Bakema, 1956; Stegeman, 1975; Talley et al., 1984; Kumar & Mukherjee, 1989; Uppal & Verma, 1990), hormones (Bielinska-Czarnecka & Bialek, 1976; Hemberg, 1985), malic and citric acids (Rumpf, 1972; Reust & Aerny, 1985), vitamins (Augustin et al., 1978; Keijbets & Ebbenhorst-Seller, 1990) and lipids (Chérif, 1973; Spychala & Desborough, 1990; Kumar & Knowles, 1993).

However, from these results it is still difficult to depict a global picture of the metabolism of potato tubers during storage. The main difficulty encountered in this

kind of study is because the biochemical changes occurring during storage of potato tubers depend not only on the storage conditions (temperature, relative humidity, light, etc.) but also on the history of the tubers before harvest (Burton, 1989). Moreover, none of the above biochemical parameters is a global indicator of the metabolism.

In contrast, ATP, which is a key molecule in all energy transfer reactions in cells, might be a good candidate. It is worth mentioning that, during the growth of potato tubers, the ATP content undergoes important changes (Fanta et al., 1988). In order to measure the ATP content in potato tubers an apparatus (the prototype NIVAC 260) was built which allowed us to extract and measure ATP automatically not only in potato tubers but also in several other biological materials (onions, apples, pears, seeds, milk, etc.).

In this investigation, we report on the characteristics of the method which was developed for this apparatus, which can also be used manually, and give the first results obtained with two cultivars of potato tubers stored under controlled conditions. Some of these results have been presented elsewhere (Biotto et al., 1988a,b).

Materials and methods

Potato cultivars and storage conditions. Three cultivars were used: Ukama, Granola and Bintje which have been described by Reust (1991) and were provided by the Station fédérale de Recherches agronomiques de Changins (Switzerland). Seed potato tubers were stored in darkness at 15 °C and in 80–90% RH. Sprouting and tuberization are defined according to Reust (1986).

Reagents. Apyrase (ATP:ADP diphosphohydrolase), Sigma grade 1, E.C. 3.6.1.5; ATP (disodium salt) and firefly lantern extract (luciferine-luciferase) were provided by Sigma; ethanolamine was supplied by Fluka (Switzerland).

Preparation of potato extracts. Potato tubers were washed carefully with tap water, cut into small pieces, then ground in a Waring blendor containing the extraction solution proposed by Maire (1984), consisting of 52.6 mM MOPS (3-[N-morpholino]propane sulfonic acid) and 0.6 N sulfuric acid (250 to 500 mg potato fresh weight/ml of final volume). The acidic homogenate was heated for 5 min at 60 °C, diluted 1:30 with the extraction solution, and then ground a second time (1 min at full speed) in a polytron Type 87 PTA 10S Kinematica (Lucerne, Switzerland) and filtered (Schleicher and Schüll, 5122). An aliquot of 1 ml of the filtrate was mixed with 19 ml of a neutralization solution containing 3.2 mM EDTA-Na_2 (ethylenediaminetetraacetate-Na_2), 3.5 mM $MgCl_2$, 52.6 mM MOPS (3-[N-morpholino]propanesulfonic acid) adjusted to pH 9 with ethanolamine. The final pH of the extract was 7.3±0.1. In the results shown in Fig. 5, the sampling was made by collecting the fourth of each tuber (out of 15) by cutting them two folds longitudinally (apical-basal direction).

Determination of ATP content. ATP content was measured by bioluminescence : 1 ml of potato extract was mixed in the cuvette of the Skan XP-2000 luminometer with 0.2 ml of firefly lantern extract (FLE 250, diluted to 60 ml with distilled water).
After the addition of FLE, the light signal was integrated for 5 s by the luminometer and compared with standards of ATP (0 to 100 nM).

Measurement of apyrase activity. A fixed amount of commercial apyrase from potato tubers (0.1 ml containing 10 Sigma units) was mixed with 0.9 ml of the extraction solution, and heated at various temperatures (40.5±0.5, 48.5±0.5 and 59.5±0.5 °C) for 5 min. After heat treatment, 18 ml of the neutralization solution was added to reach pH 7.3 and 1 ml of ATP (10 µM of ATP in the neutralization solution). The reaction mixture was incubated at 20 °C and the residual ATP was measured as a function of time.

Results

As can be seen in Fig. 1, the ATP content in potato tubers increased progressively as a function of fresh weight but in a non-linear fashion. When exogenous ATP (18 nmol) was added in the acidic extract, no significant loss was observed whatever the amount of fresh weight was used. However, these results indicate that during the experimental procedure, a certain amount of ATP was degraded. This might be due to (a) hydrolysis of ATP in the acidic medium; (b) an enzymatic hydrolysis of ATP which occurred after the neutralization of the acidic filtrate or (c) an incomplete extraction of ATP from potato tubers. The first proposal can be discarded since a degradation of ATP was never observed in ATP standards under our current experimental conditions (results not shown, but see results in Fig. 2B).

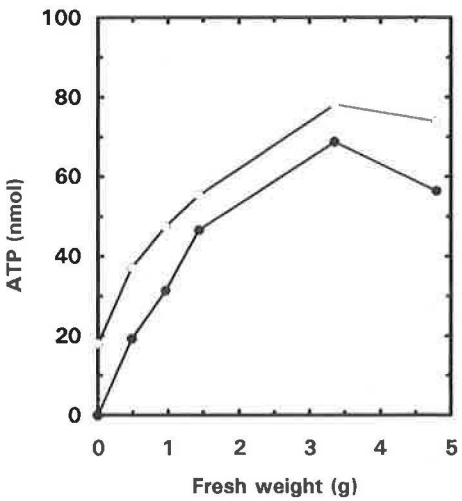

Fig. 1. ATP content in potato tubers (cv. Bintje) as a function of fresh weight. Controls (–●–); samples supplemented with 18 nmol of ATP (–○–). The acidic filtrate was not heated.

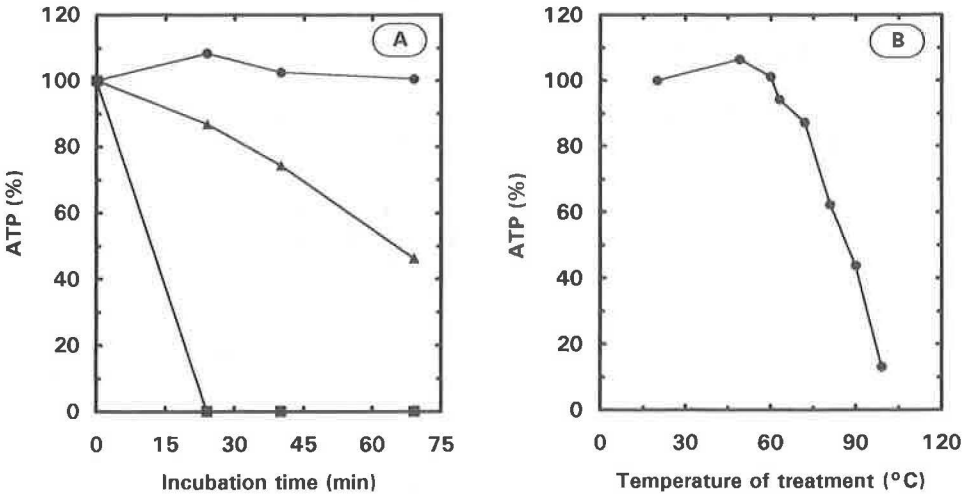

Fig. 2. Effect of various temperatures on the Apyrase activity (A) and on ATP stability (B). A: the acidic apyrase solution was heated for 5 min at 40.5 (-■-), 48.5 (-▲-) or 59.5 °C (-●-). B: an acidic solution of ATP (2 nM) was heated at various temperatures for 5 min, then neutralized.

The second hypothesis was suggested by several reports showing that potato tubers contain a very active ATPase (Anich et al., 1990) which is stable in H_2SO_4 0.6 N (Biotto et al., 1988b). Fig. 2A shows that when commercial apyrase was heated, its further activity depended greatly on the temperature during heating. For instance, at 40.5 °C the hydrolysis of ATP was very fast whilst at 59.5 °C the hydrolytic activity was completely obliterated. Thus, in order to prevent the enzymatic degradation of ATP, it was essential to heat the acidic potato tuber extracts at least to 60 °C. Fig. 2B shows that under these experimental conditions, ATP was not degraded. Indeed, in heating the potato tuber extract at 60 °C the ATP content was linearily correlated with the amount of fresh weight extracted, without apparent loss of the adenine nucleotide (Fig. 3). Beyond 3.5 g of fresh weight, extracts became slightly opalescent causing a quenching of the luminescence measured in the firefly assay. In addition, no loss of the exogenous ATP occurred during the procedure.

In order to study the variations of ATP content in several cultivars of seed potato tubers, it was necessary to store a great number of samples at low temperature (-25 °C) before ATP analyses. Thus, it was necessary to verify whether ATP was stable under such conditions. In Fig. 4A, it can be seen that at -25 °C a solution of ATP was not stable and its level decreased exponentially as a function of time. The half decrease in ATP level occurred after approximately 12 days. From the exponential curve, it was possible to calculate a constant (similar to that of the radioactive disintegration constant) expressing the hydrolysis of ATP as a function of time, as indicated in the legend of Fig. 4A. This hydrolysis constant decreased in a linear fashion when the fresh weight of potato tubers was increased, showing that in order to prevent the hydrolysis of ATP during storage it was advantageous to prepare acidic potato

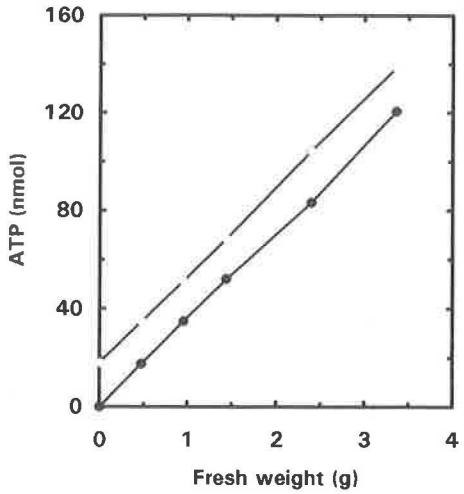

Fig. 3. ATP content in potato tubers (cv. Bintje) as a function of fresh weight. Controls (–●–); samples supplemented with 18 nmol of ATP (–○–). The acidic filtrates were heated at 60 °C for 5 min.

homogenates containing at least 500 mg fresh weight/ml (Fig. 4B). In addition, it is noteworthy that the extrapolation of the hydrolysis constant values to zero fresh weight (Fig. 4B) corresponded to the value calculated for a pure solution of ATP (see legend of Fig. 4A).

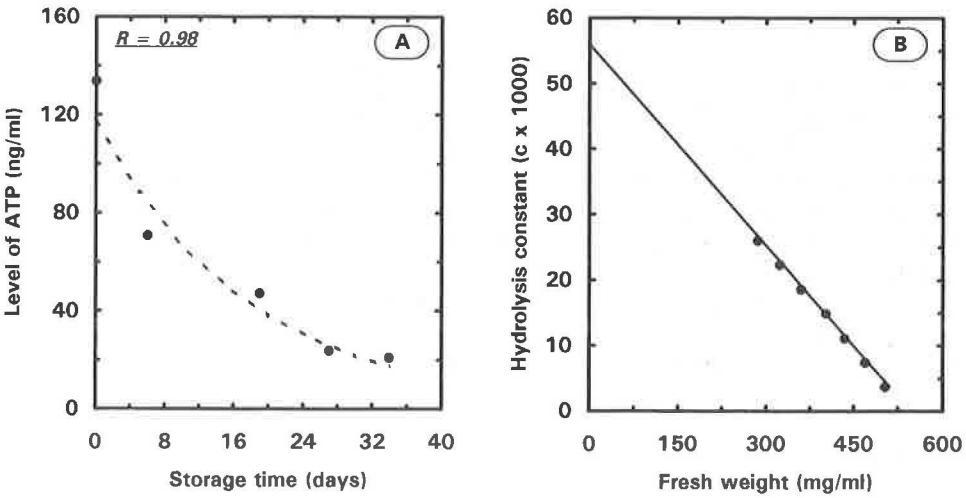

Fig. 4. A: Kinetics of the decrease in ATP level during storage at -25 °C of a pure ATP solution in an acidic medium (0.3 N H_2SO_4, 52.6 mM MOPS). The decay (---) is best fitted by the exponential form: $[ATP]_t = [ATP]_{to} \times e^{-c(t-to)}$, where c is the hydrolysis constant; c = 0.056[day^{-1}].
B: Linear dependence of the ATP hydrolysis constant on the concentration of potato extract (mg fresh weight/ml) in acidic medium stored at -25 °C.

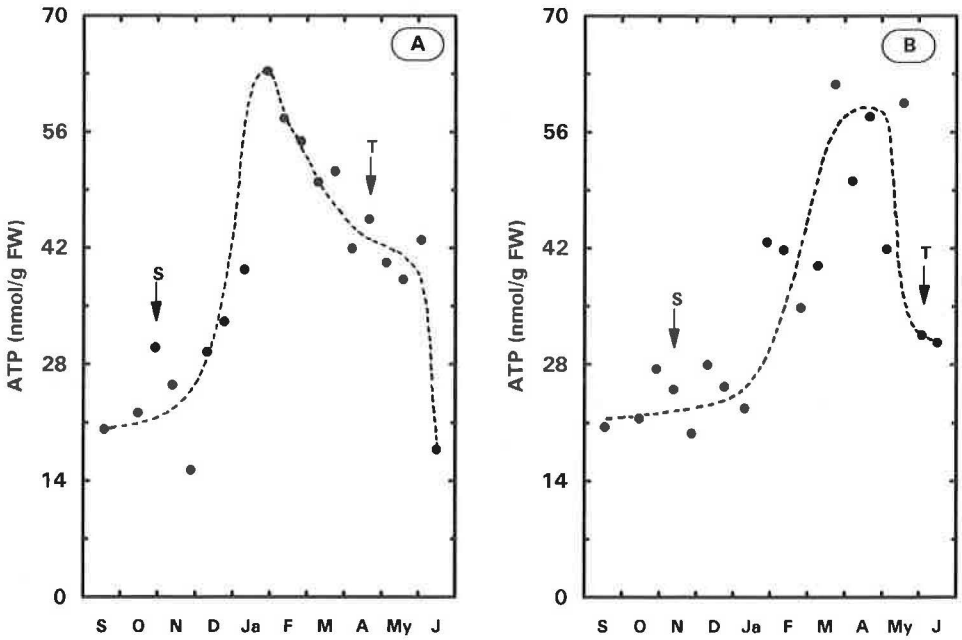

Fig. 5. Variations of ATP content in seed potato tubers stored at 15 °C, in darkness and at 80 to 90% RH, from September (S) 1986 to June (J) 1987. A: cv. Ukama; B: cv. Granola. Each point represents the average of at least 3 determinations with a standard deviation <5%.
S = sprouting; T = tuberization

Taking all the precautions described in the above method, we have determined the variations of ATP content in two seed potato tubers (e.g. cv. Ukama and cv. Granola) during their storage (darkness; 15 °C; 80–90% RH). In Fig. 5 it can be seen that during storage the ATP content of potato tubers underwent great changes. For cv. Ukama, a peak (63 nmol/g fresh weight) was observed at the end of January (Fig. 5A). In contrast, for cv. Granola the highest values (56 nmol/g fresh weight) were reached at the end of March and remained more or less constant until the middle of May (Fig. 5B). The tuberization (see T in Fig. 5) occurred at the end of April for cv. Ukama and at mid-June for cv. Granola. At this physiological stage the ATP content, compared to its highest value, dropped by about 35 to 50%. During the dormancy of seed potato tubers the level of ATP stayed at low values (around 24 nmol/g fresh weight). The ATP content began to increase after the break of dormancy which occurred in November for both cultivars (see S in Fig. 5).

Discussion

Over the past 20 years there have been several attempts to find biochemical parameters which could pinpoint the physiological state of postharvest aging in fruits and vegetable. For seed potatoes, in particular, the physiological stage during storage is estimated generally by the growth and morphological characters of sprouts (Madec

& Pérennec, 1955; Kawakami, 1962; Krijthe, 1962; Hartmans & van Loon, 1987). Although sprout growth reflects internal changes in the stored tuber and affects the whole life cycle of plant, there are large differences between cultivars in sprout growth and tuber aging (Reust & Aerny, 1985). Therefore, it is highly desirable to quantify the physiological stage of tubers by one or several biochemical parameters expressing the metabolic state, so that growers and seed companies can optimize storage conditions for individual cultivars (Reust & Aerny, 1985).

Preliminary experiments have shown that the ATP level of onions, apples, pears and seeds undergoes great changes during storage (Biotto et al., 1988a; Siegenthaler & Douet-Orhant, unpublished). However, the method used could not be applied without modification for potato tubers (Fig. 1). This prompted us to develop a method specific for this vegetable.

Usually the extraction of ATP from biological material is carried out in the presence of trichloracetic acid (Lundin & Thore, 1975). However, this extractant presents several drawbacks: (1) its toxicity, (2) the necessity to work at a low temperature during the extraction procedure to avoid the degradation of ATP and (3) the absolute requirement to remove trichloracetic acid after the extraction in order to prevent the precipitation of luciferase during the bioluminescence assay. Therefore, H_2SO_4 was chosen as the extractant which is as efficient as trichloracetic acid and presents, to a much lower degree, the above-mentioned drawbacks (Maire, 1983). However, in contrast to trichloracetic acid, sulfuric acid inactivated the apyrase, without destroying or precipitating it, and its activity was restored after neutralization (Fig. 2). In order to prevent the enzymatic hydrolysis of ATP in neutralized potato tuber extracts, it was necessary to heat the acidic extract at 60 °C for 5 min (Fig. 2A) and to verify that ATP was stable at this temperature (Figs 2B and 3). Moreover, to avoid a loss of ATP during the conservation of acidic extracts at -25 °C, a preparation of at least 500 mg fresh weight per ml was a prerequisite (Fig. 4A and B).

In summary, the proposed method presents the following advantages: (1) it is simple and rapid; (2) it is reliable and sensitive; (3) it allows the preparation, the conservation and the measurement of a great number of samples; (4) it can be used on a routine basis by non-specialists in biochemistry; (5) it can be carried out manually or automatically, as with the prototype NIVAC 260.

This method has been applied successfully to the changes in ATP levels occurring during storage of potato tubers. These first results show the following features.

Firstly, the ATP level varied markedly and in a non-uniform way in stored potato tubers (Fig. 5) in contrast to other biochemical parameters, such as malate, citrate, sucrose (Rumpf, 1972; Reust & Aerny, 1985; Lisinka & Aniolowski, 1990) and lipid peroxidation (Kumar & Knowles, 1993) which undergo uniform changes devoid of marked minima or maxima.

Secondly, sprouting and tuberization were accompanied by typical changes in the level of ATP (Fig. 5). Indeed, in both cultivars (Ukama, Fig. 5A; Granola, Fig. 5B), the dormancy was characterized by a low amount of ATP, followed by an increase in ATP level which corresponded to the onset of sprouting. By contrast, the beginning of tuberization was preceeded by a drop in ATP content. Thus, the ATP level was

correlated to the physiological periods which characterize potato tubers during storage and may be of potential interest to potato growers.

Thirdly, the marked alterations of the energetic metabolism which take place during the storage period (Fig. 5; Müller, 1975; Mikitzel & Knowles, 1990; Mikitzel, 1990) reflect the anabolic/catabolic balance of the potato tuber. Whereas ATP formation can be mediated by oxidative phosphorylation (Dizengremel, 1985; Mikitzel & Knowles, 1990), glycolysis and/or *de novo* biosynthesis, ATP diminution may be due to ATP utilization and/or enzymatic degradation (Fanta et al., 1988; Anich et al., 1990). This reflects the complexity of the reactions occurring during the storage period which could be partially solved by studying other bioenergetic-linked parameters and their distribution in the apical and basal parts of the tuber.

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