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Note

Chromatographic separation and identification of short-chain acid-soluble polyphosphates from *Saccharomyces cerevisiae*

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Several investigators have reported the presence of a large variety of inorganic linear polyphosphates (polyPs) in procaryotic[1,2] and eucaryotic organisms[3,4]. Such compounds are present in cells in the form of an acid-soluble fraction ranging in chain length between two (pyrophosphate) and several hundred (for a review see ref. 5) and an acid-insoluble fraction of higher molecular weight, complexed with RNA and proteins. The acid-soluble polyPs can be partially characterized by paper chromatography[6,7], but by this method only products with chain lengths of 2 or 3 can be completely separated. A complete separation of the different short-chain polyPs can be achieved by making use of various types of thin layers and various solvents[8,9]. These methods have the disadvantage of not allowing the separation of polyP from other phosphorylated compounds, such as nucleotides and nucleotide coenzymes. They are therefore not really suited for the study of polyPs in extracts obtained from living cells. A separation of polyPs from other phosphorylated compounds can be achieved by making use of the thin-layer chromatographic (TLC) technique introduced by Cashel[10] for the separation of phosphorylated guanosine nucleotides. With this method, Ludwig *et al.*[11] have resolved three acid-soluble phosphorylated compounds, two of which were tentatively identified as tri-polyP and tetra-polyP. In this paper we report an improvement of this one-dimensional chromatographic technique which permits the study of polyPs in cell extracts up to a chain length of 8. A subsequent modification of our technique made it possible to identify by coloration the various polyP polymers on the thin-layer chromatograms.

MATERIALS

Preparation of extracts containing ^{32}P-labelled acid-soluble polyPs and nucleotides was carried out according to Ludwig *et al.*[11] from *Saccharomyces cerevisiae* yeast cultures grown in a low phosphate medium[12] in the presence of 50–100 μCi/ml carrier-free ^{32}P-orthophosphate. Cell samples were diluted with an equal volume of ice-cold 2 *N* formic acid and extracted at 0° for 30 min, after which they were centrifuged at 5000 rpm for 10 min. The supernatant containing the acid-soluble material was stored at −20° for further analysis.

Carrier-free $H_3{}^{32}PO_4$ was obtained from the Radiochemical Centre, Amersham, Great Britain. Standard ^{31}P polyphosphates used for colorimetric determination were

from Sigma, St. Louis, Miss., U.S.A. Separation and identification of polyPs were achieved by making use of Polygram cell 300 PEI plates from Macherey, Nagel & Co., Düren, G.F.R., and Kodak XR-5 X-ray films.

All reagents and non-radioactive standard nucleotides were of the highest purity available from commercial sources.

METHODS

One-dimensional ascending chromatography

In order to detect the soluble polyPs contained in yeast extracts we have modified the method of Cashel[10] for the chromatographic assay of polyphosphorylated nucleotides and have resolved six polyP polymers. A sheet of filter paper 15 cm long was stapled to the upper edge of the chromatographic plates and up to 100 μl of radioactive material were spotted 1.5 cm from the bottom of the plate. 0.2 μmoles of ATP and of GTP were spotted together with the radioactive material, and the migration of these compounds was followed with a UV lamp. Ascending chromatography was performed using 1.5 *M*, 2.2 *M* and 2.5 *M* NaH_2PO_4, pH 3.4, for 12, 25 and 28 h, respectively. All the material with a relatively high mobility was collected on the filter paper which was detached and discarded after chromatography. The plates were then exposed to X-ray films and were developed to visualize the radioactive spots.

Two-dimensional chromatography

0.5 μmoles of each available standard polyP dissolved in 1 *N* formic acid was applied to a chromatographic plate prepared as described above, together with 20–50 μl of radioactive material from yeast cultures, and with 0.2 μmoles of GTP. The samples were eluted in the first dimension with 2.5 *M* NaH_2PO_4, pH 3.4, for 25 h, after which the filter paper was removed and the plates thoroughly washed in methanol. Without drying, the plates were washed in distilled water and then dried under a flow of cold air. After rotation for the second dimension, a second sheet of filter paper was applied to the top of the plates and chromatography was performed in 2.7 *M* ammonium formate containing 4.2% boric acid and adjusted to pH 7.0 with NH_4OH. In the course of this elution step the migration of GTP was monitored with an UV lamp and elution was terminated when the GTP had run off completely onto the filter paper. The time required for this was *ca.* 24 h. By means of this procedure the ammonium formate solution removed from the plates practically all the inorganic phosphate used as the first solvent, which would interfere with coloration of ^{31}P polyPs.

Coloration method

For this purpose each plate was dipped in a solution consisting of ammonium molybdate (1 g) dissolved in water (8 ml) and perchloric acid (3 ml; *ca.* 70%) and diluted with acetone to 100 ml. After 1–2 min the plates were allowed to dry for 7–8 min at 85°. With this hydrolytic method[13] the inorganic phosphates react with the ammonium molybdate giving rise to yellow-green spots on a blue background. By comparing the two-dimensional chromatograms coloured as above with their

companion autoradiogram and looking for coincident spots, it was possible to identify the polyPs synthesized in yeast cultures.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of phosphorous-containing compounds obtained by one-dimensional TLC of an acid-extract of yeast cells, using either 1.5 *M*, 2.2 *M* or 2.5 *M* sodium phosphate buffer.

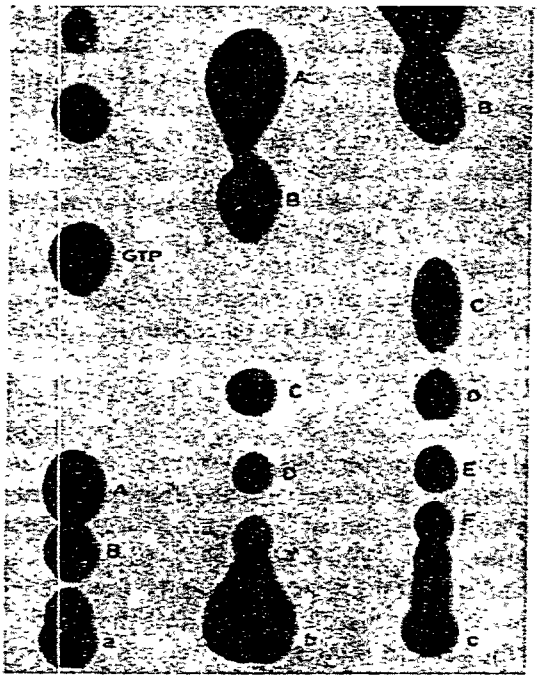

Fig. 1. Autoradiographs of three one-dimensional chromatographic separations on PEI-cellulose of ^{32}P-labelled polyphosphates from yeast cells. The plates were developed at pH 3,4 in: (a) 1.5 *M* phosphate buffer for 12 h; (b) 2,2 *M* phosphate buffer for 25 h; (c) 2.5 *M* phosphate buffer for 28 h. Spots A to F represent low-molecular-weight polyphosphates of chain lengths increasing from 3 to 8.

After a 12-h elution with 1.5 *M* NaH_2PO_4 all nucleotides except GTP have run off the thin-layer plates, leaving GTP and two more slowly moving compounds well resolved on the plate. It was shown by Cashel *et al.*[14] that pyrophosphate co-migrates with GTP in this type of chromatographic system. The two compounds that migrate more slowly than GTP, and which we have called spots A and B, correspond to those which have been tentatively designated by Ludwig *et al.*[11] as tri- and tetra-polyP, respectively. If one increases the elution time (25 h) and the molarity of the phosphate buffer, the GTP disappears from the plates and three (2.2 *M* buffer) or four (2.5 *M* buffer) new radioactive spots issue from the origin. Furthermore, allowing the faster-moving material to run off onto the filter paper leaves the top of the plates clean and

all the spots perfectly visible. In the following, these spots will be referred to as spots A to F, in order of decreasing mobility.

In order to demonstrate that the compounds resolved by this method were in fact polyPs and to determine their chain length, we could not analyse the spotted material directly since the concentration of material in each spot was very low. Thus, the radioactive material was allowed to co-migrate with commercial preparations of ^{31}P polyPs which could be identified by coloration. However, the phosphate remaining on the plate after elution with phosphate buffer reacts with the ammonium molybdate used for detection of the phosphoric groups giving rise to a very dark background of phosphomolybdate complexes. In order to remove the residual phosphate from the plates, after the first dimension, we decided to subject the plates to a second elution step in ammonium formate in the other dimension. This step does not have, in this case, the purpose of improving the resolution of the compounds under study, but merely that of washing out contaminating material. We therefore spotted on each plate a mixture containing the radioactive yeast preparation, GTP and one of the following commercial polyphosphates: tri-polyP, type 5-polyP (average chain length 5.5) and type 15-polyP (average chain length 14). After chromatography and autoradiography each chromatogram was colored as described in Methods and compared with the companion autoradiogram. The results of these experiments are displayed in Fig. 2 and show that spots A to F are not greatly displaced by elution in the second dimension and that the migration of these spots is roughly proportional to their migration in the first dimension. Fig. 2(a) represents the patterns obtained by autoradiography (solid line) and coloration (shading) of the mixture containing the radioactive yeast extract and ^{31}P tri-polyP. This experiment shows that the standard tri-polyP co-chromatographs with spot A, which strongly indicates that the six com-

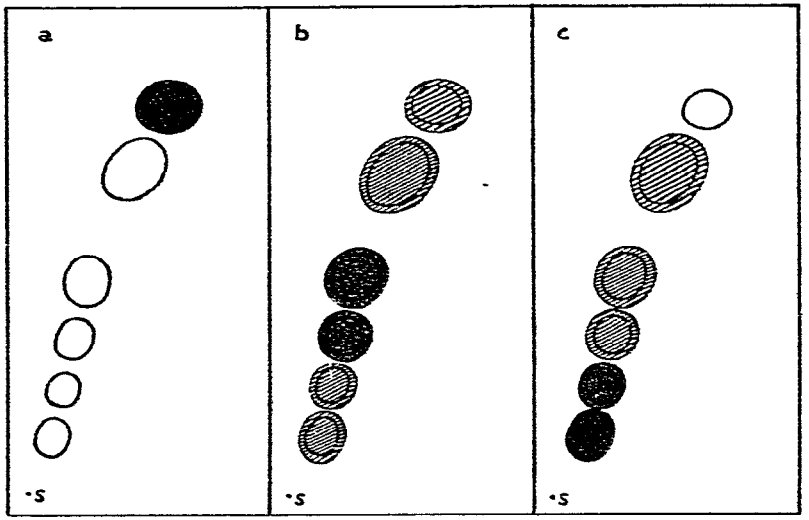

Fig. 2. Two-dimensional chromatography and identification by means of coloration of polyphosphates extracted from yeast cells as described in "Material and Methods". Additions: (a) standard tri-polyP; (b) type 5-polyP; (c) type 15-polyP. The spots encircled by solid lines correspond to polyP compounds in yeast extract visualized by autoradiography. The shaded areas are the coloured spots obtained with the standard polyPs. The intensity of shading reflects that of coloration.

pounds resolved by us have chain lengths ranging, respectively, from three to eight. Fig. 2(b) shows the results of a similar experiment performed with ^{31}P type-5 polyP. The most intensely coloured spots are those corresponding to spots C and D, which assigns chain lengths of five and six to these spots, in view of the fact that type 5-polyP has an average chain length of 5.5. Considering that spot B was found between tri-polyP and penta-polyP, it may thus be definitely concluded that it has a chain length of four. Spots A, B, C and D have thus been identified as linear polyphosphates having chain lengths of three, four, five and six, respectively. From their chromatographic behavior, it can be surmised that the remaining two spots, E and F, have chain lengths of seven and eight.

The results of an experiment performed with type 15-polyP, Fig. 2(c), show that this product does not contain a detectable amount of tri-polyP but contains tetra- and penta-polyP in small amounts, and other polyphosphates of higher molecular weight in larger amounts. However, some of the compounds of higher molecular weight do not migrate from the origin where the most intense yellow colour is to be found. When the coloration method described above was used in conjunction with standard trimeta- and tetrameta-polyP, the results were not reproducible; it was clear, however, that these products migrated in areas distant from those occupied by the linear PolyPs.

These studies have thus made it possible to resolve on one-dimensional chromatograms and to identify six polyP polymers present in extracts of *S. cerevisiae*. The method is easy and practical and lends itself well to quantitative studies of the intracellular concentration of these compounds under various *in vivo* conditions.

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