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A Specific Micromethod for the Determination of Phytic Acid in Biological Material

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A method for the quantitative determination of phytic acid in biological material is described. The method, permits a determination of phytic acid in quantities below 0.1 mg even if the material contains closely related compounds including *myo*-inositol pentakisphosphate.

(Keywords: Inositolphosphates; Microdetermination; Paper electrophoresis; Phytic acid)

Eine spezifische Mikromethode für die Bestimmung von Phytinsäure in biologischem Material

Es wird eine Methode zur quantitativen Bestimmung von Phytinsäure in biologischem Material beschrieben. Die Methode erlaubt die Bestimmung von Phytinsäure in Mengen von weniger als 0.1 mg, selbst wenn das Untersuchungsmaterial nahe verwandte Substanzen wie z.B. *myo*-Inositpentakisphosphat enthält.

Introduction

In the literature we can find several methods elaborated for the isolation and the quantitative determination of phytic acid in biological material[1–7]. However, most of these methods allow only for the determination of phytic acid in relatively large amounts and none of them is suitable for the determination of phytic acid in the presence of closely related compounds like *myo*-inositol 1,3,4,5,6-pentakisphosphate, a substance to be found in chicken erythrocytes, where it regulates the O_2-affinity of hemoglobin in a similar manner as 2,3-bisphosphoglycerate in mammalian erythrocytes. In the present paper we describe a method for the determination of phytic acid down to quantities below 0.1 mg, which can also be used when natural compounds very closely related to phytic acid are present in the material to be analyzed.

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Materials and Equipment

All reagents used were analytical grade. Na_2SO_3, 30% H_2O_2, H_2SO_4, NaOH, oxalic acid, ammonium heptamolybdate and *L*-ascorbic acid were purchased from E. Merck, Darmstadt; phytic acid was obtained from Sigma. For the paper electrophoresis Whatman 3M paper was used.

The paper electrophoresis was carried out in the apparatus according to *Wieland* and *Pfleiderer*, obtained from Messrs. Hormuth, Heidelberg. For photometric measurements a Gilford spectrophotometer was used. Inorganic phosphate was determined according to *Chen* et al.[8]; for qualitative detection of phosphate-containing compounds on the paper after electrophoresis the reagent of *Harrap*[9] was used.

Description of the Method

The biological material to be analyzed (usually 3-10 g) is frozen with liquid nitrogen and then carefully ground in a cooled mortar. The ground product is boiled with acetone under reflux to remove lipids, in the case of plant material also including chlorophyll. The solid residue is collected by filtration, washed several times with acetone, and eventually soaked dry. Then the remaining powder is carefully extracted under stirring with 25 ml 4*N*-HCl at room temperature for 30 min; this procedure is repeated three times. The combined extracts are evaporated to dryness, dissolved in water and the water evaporated again; this is repeated until the excess of HCl is removed from the aqueous solution. The phosphate-containing compounds are adsorbed on Dowex 1×4 (OH^--form) in a column 1×15 cm. This column is washed with at least 200 ml water. The phosphate-containing compounds are then eluted with 150 ml 4*N*-HCl. Then the eluate is evaporated to dryness and several times again evaporated with water to remove HCl. The remainder is once again dissolved in water an aliquot of the solution, which besides phytic acid contains various phosphoesters and inorganic phosphate, is then subjected to high voltage paper electrophoresis in a 0.1 *M*-sodium oxalate buffer, *pH* 1.5. This puffer was prepared by titrating a 0.1 *M*-solution of oxalic acid with 0.1 *M*-NaOH until the wanted *pH* value was reached. In comparison with other buffers commonly used, this buffer solution has the advantages that separation of phosphorylated compounds is very good and that the spots of the substances on the paper remain concentrated. Before electrophoresis the paper (50×25 cm) is soaked in the buffer solution and then slightly dried before being put into the electrophoresis chamber. Now the test solution (up to 400 µl) is applied on the starting line. The electrophoresis is carried out at 700-800 V and 100-140 mA for 2.5 h at 0 °C. 2,4,6-Trinitrophenol, applied on both sides of the starting line, is used as an easily visible reference substance; its distance of migration should be at least 25 cm and is arbitrarily set as 1. The distances of migration of

several phosphorylated compounds in relation to that of 2,4,6-trinitrophenol are given in Table 1. In order to determine the area that contains phytic acid and therefore should be cut out of the paper, a standard of this compound is also applied on both sides of the starting line.

After electrophoresis the paper is dried, the standard area cut off on both sides and developed according to *Harrap*[9]. The zone of phytic acid according to the standards is cut out, adding 1 cm in the direction of migration to make sure that all phytic acid is eluted.

Table 1. *Distances of migration of myo-inositol, inorganic orthophosphate, and several phosphorylated derivatives of myo-inositol in relation to the distance of migration of 2,4,6-trinitrophenol under the conditions of paper electrophoresis as described in this paper*

Compound	Distance of migration
2,4,6-Trinitrophenol	1
myo-inositol	0.22
inorganic phosphate	0.72
myo-inositol monophosphate	0.92
myo-inositol bisphosphate	1.20
myo-inositol trisphosphate	1.45
myo-inositol 1,3,4,5,6-pentakisphosphate	1.95
phytic acid	2.08

The following procedures should be carried out under the hood. The part of the paper containing phytic acid is extracted 4 times, each time with 25 ml warm water (60 °C); the extracts are combined and evaporated to dryness. Then the sample is boiled with 1.5 ml conc. H_2SO_4 and 2 ml H_2O_2; after 10, 20 and 30 min 2 ml H_2O_2 are added to replace the H_2O_2 destroyed during the process; the boiling is continued until H_2SO_4 just begins to evaporate. After cooling 0.5 g solid Na_2SO_3 is added, which causes the development of SO_2 and simultaneously the solidification of the sample. This is then dissolved in 2-3 ml water and boiled again just until the evaporation of H_2SO_4 begins. The sample is then diluted with water to 25 ml and kept on a hot waterbath to hydrolyze polyphosphate that might have been formed during the preceding treatment.

5 ml of the solution thus obtained are treated at 37 °C with 2 ml freshly prepared reagent of *Chen* et al., consisting of 2 parts water, 1 part 3 *M*-H_2SO_4, 1 part 2.5% ammoniumheptamolybdate, and 1 part 10% *L*-ascorbin acid. The absorbance at 820 nm is measured. Preferably

a calibration curve prepared with highly purified phytic acid (range from 0.05 to 400 μmoles), treated with electrophoresis degradation as described above, should be used. The loss of phytic acid phosphate during the complete procedure is consistently $12 \pm 1\%$. If slighly less exact values are sufficient, a calibration curve obtained with inorganic phosphate might also be used.

Remarks

Carried out in the way described above the method is highly reproducible, as has been shown by adding known amounts of phytic acid to plant tissues and measuring the recovery of the substance by our method.

It has to be pointed out that the method is very sensitive to phosphate; therefore all reagents should be completely phosphate-free and only bidistilled water should be used. The degradation of phytic acid by treatment with H_2SO_4 and H_2O_2 is complete. The following reduction of the sample with Na_2SO_3, by preventing the ascorbic acid of the reagent mixture to be destroyed by oxidation products of paper residues, increases the sensibility and reproducibility of the colorimetric determination.

Under the condition described electrophoresis permits to separate phytic acid from all other inositolphosphates and also from relatively high amounts of inorganic phosphate. Even in the presence of other phosphorylated inositol derivatives in plant material, quantities of phytic acid below 0.1 mg can be quantitatively recovered and determined. A quantitative separation of phytic acid from *myo*-inositol 1,3,4,5,6-pentakisphosphate is possible if the concentration of the latter compound is lower than that of phytic acid; if necessary this condition can be fulfilled by adding known amounts of phytic acid to the sample to be analyzed. Overloading of the paper must be avoided in all cases.

The reported method has been used for an investigation of the phytic acid content of a higher plant under differing metabolic conditions[10]; very consistent results were obtained.

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References

1 *D. Oberleas*, Methods Biochem. Anal. **20**, 87 (1971); a review of methods reported until 1970.

2 *U. S. Kutikova, S. A. Babakhodzaeva*, and *A. Madzhidov*, Khim. Farm. Zh. **6**. 57 (1972).

[3] *Kh. Z. Brainina*, *N. D. Fedorova*, and *L. S. Fokina*, Zh. Anal. Khim. **29**, 254 (1974).
[4] *B. F. Harland* and *D. Oberleas*, Cereal Chem. **54**, 827 (1977).
[5] *C. K. Hong* and *I. Jamana*, Nippon Doho-Hirogaku Zasshi **47**, 153 (1976).
[6] *R. Ellis*, *E. R. Morris*, and *C. Philpot*, Anal. Biochem. **77**, 536 (1977).
[7] *D. Inhülsen* and *R. Niemeyer*, Z. Pflanzenphysiol. **88**, 103 (1978).
[8] *P. S. Chen*, *T. Y. Toribara*, and *H. Warner*, Anal. Chem. **28**, 1756 (1956).
[9] *F. E. G. Harrap*, Analyst **85**, 452 (1960).
[10] *O. Scheiner*, *F. Pittner*, *O. Bollmann*, and *R. Kandeler*, Z. Pflanzenphysiol. **88**, 295 (1978).