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Time course of formation of inositol phosphates during enzymatic hydrolysis of phytic acid (myo-inositol hexaphosphoric acid) by phytase determined by capillary isotachophoresis

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Abstract

Capillary isotachophoresis with conductivity detection was applied to the investigation of the hydrolytic decomposition of phytic acid (myo-inositol hexaphosphoric acid) by phytase, and for the formation of the reaction products as a function of time. The quantitation of all analytes (besides phytic acid the mono- to penta-phosphorylated inositols and orthophosphate) can be carried out using two different buffer systems.

1. Introduction

The determination of the time course of the enzymatic hydrolysis of phytic acid (myo-inositol hexaphosphoric acid, IP6) has been carried out so far by an indirect method which is based on the measurement of the concentration of Fe(III) remaining in solution after the formation of an insoluble Fe–IP6 complex [1]. Fe(III) is then determined photometrically as rhodanide complex. This indirect measurement was necessary because IP6 has neither a specific reagent nor a characteristic absorption spectrum for direct determination, as pointed out in this paper.

It is obvious that the indirect method is subject to a number of errors, because not only phytic acid, but also its degradation products may react with the Fe ions due to their chemical similarity to IP6. This method is unable to quantify these particular compounds, which is a prerequisite for the study of the reaction kinetics. Therefore a method has been introduced by the same authors [1] which allows the determination of all the compounds of interest, namely ^{31}P NMR. Nevertheless, this method has a number of drawbacks. Besides its large instrumental and chemical expenditure (e.g. $^{2}H_2O$ is used as a solvent for NMR measurements) no separation of the solutes is carried out, leading to superimposed signals which limit their relevance.

Isotachophoresis (ITP) with conductivity detection seems to fulfill all the demands required for the fast, simple and reliable analysis of phytic acid and its hydrolysis products (cf. e.g. [2]): it is

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a separation method of high performance for ionic components, which enables the resolution of chemically similar compounds. It combines the separation and the detection principle for these ionic separands, both based on the effective mobilities, which leads to a more or less universal detectability for these analytes, and makes the detection independent of individual properties such as UV absorbance.

ITP has been applied for the direct determination of phytic acid in food [3], where the preseparation from the lower inositol phosphates (penta, IP5 to mono, IP1) and orthophosphate (P) was a necessity. In the present paper it is applied to separate the inositol derivatives with different degrees of phosphorylation, making it possible to observe the formation and decay of the degradation products of phytic acid during the hydrolysis with phytase.

2. Experimental

2.1. Chemicals

The following chemicals were used: hydrochloric acid (analytical-reagent grade; Merck, Darmstadt, Germany), 2-morpholinoethanesulfonic acid (99%), phytic acid (dodecasodium salt), 1,3 - bis[tris(hydroxymethyl)methylamino]propane (bis-tris-propane, 99 + %), hydroxyethyl cellulose (all from Aldrich, Steinheim, Germany); creatinine (99%), caproic acid (99–100%), phytase (crude from wheat, activity ca. 0.015 units per mg solid) (all from Sigma, Deisenhofen, Germany); ϵ-aminocaproic acid (>99%, Fluka, Buchs, Switzerland).

For the preparation of the buffers water deionised with a mixed-bed ion exchanger was used.

2.2. Apparatus

The volume-coupling instrument (Ionosep 900.1, Recman-Laboratorní Technika, Ostrava, Czech Republic) is equipped with PTFE capillaries (pre-separation part 50 × 1 mm, separation part 150 × 0.45 mm, detection part 70 × 0.3 mm) and a contactless high-frequency conductivity cell. Injection is carried out by the use of a 20-μl loop.

The constant current applied was 70 μA initially and 30 μA during detection.

2.3. Enzymic hydrolysis of phytic acid

A 50-mg amount of phytic acid (as dodecasodium salt) and 50 mg phytase were added to 50 ml buffer (0.04 *M* HCl adjusted to pH 5.15 with creatinine) and placed into a 100-ml flask. The solution was stirred on a water bath at 51°C. During the enzymic hydrolysis aliquots of the reaction mixture were taken at different times. The enzyme present in the samples was inactivated by boiling water for 1 min. Then the samples were stored at −20°C. Prior to the analysis the samples were defrosted and diluted 20-fold.

3. Results and discussion

3.1. ITP separation

In order to follow the time dependence of the hydrolytic decay of phytic acid on the one hand, and of the formation and hydrolysis of the degradation products (the lower inositol phosphates and phosphate) on the other hand the appropriate ITP conditions to separate the analytes must be found. It can be seen from Fig. 1 that system 1 (Table 1) allows the separation of most inositol phosphates and phosphate (IP4–IP1, P), but does not lead to a separation of IP6 from IP5. Therefore this system can be applied for the quantitation of these lower phosphates only. For the separation and quantitation of IP5 and IP6, however, another system has to be found. It is not likely that the separation can be enhanced by varying the pH, because it can be expected that the analytes have about the same p*K* values. For this reason the use of bis-tris-propane which has complex-forming properties is applied as counter ion of the leading electrolyte [2]. The resulting electropherograms, obtained by electrolyte system 2 (Table 1) are depicted in Fig. 2. It can be seen that IP6 and IP5, which

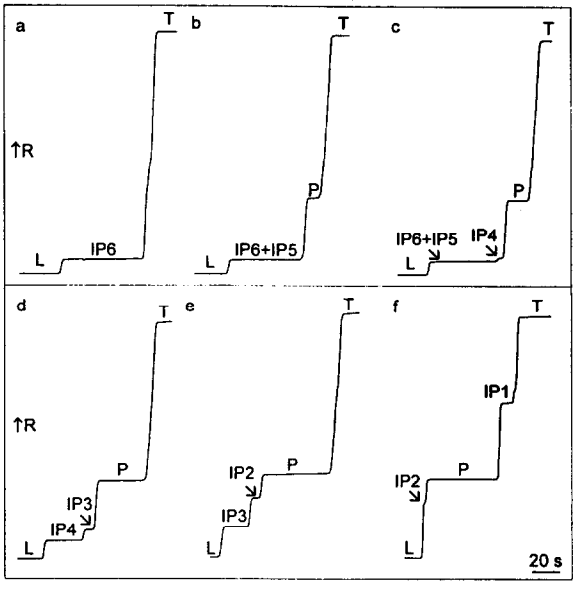

Fig. 1. Isotachopherograms of the reaction mixture of the enzymatic hydrolysis of phytic acid (IP6) after different times of reaction obtained with electrolyte system 1. IP6 and IP5 form a mixed zone. The degree of phosphorylation is indicated by the number in the symbol of the separands, e.g. IP5 for inositol pentaphosphate, etc. P = Orthophosphate; L = leading electrolyte; T = terminating electrolyte; R = electric resistance. Reaction time: (a) 0 min; (b) 2.5 min; (c) 20 min; (d) 40 min; (e) 90 min; (f) 210 min.

formed a mixed zone in system 1 are indeed migrating in two separated zones. They are also well separated from IP4 and P. It is therefore possible to quantitate all analytes by the use of these two buffering electrolyte systems.

Table 1
Buffering electrolyte systems for the determination of inositol phosphates and orthophosphate

System	Leading electrolyte[a]	pH	Terminating electrolyte
1	10 m*M* HCl + EACA	4.5	5 m*M* Caproic acid
2	10 m*M* HCl + BTP	6.1	5 m*M* MES

EACA = ϵ-Aminocaproic acid; BTP = bis-tris-propane; MES = 2-morpholinoethanesulfonic acid.
[a] Leading electrolytes contain 0.1% hydroxyethyl cellulose.

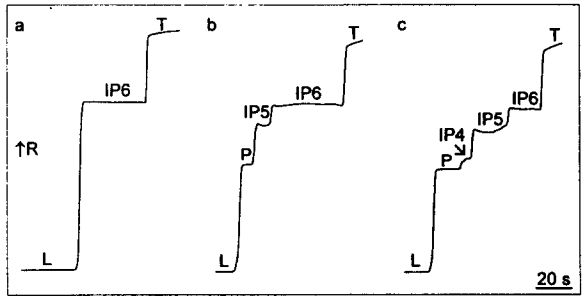

Fig. 2. Isotachopherograms of the reaction mixture of the hydrolysis of phytic acid shown in Fig. 1a, b and c, obtained with electrolyte system 2, demonstrating the separation of IP6 from IP5. Abbreviations as in Fig. 1.

3.2. Time course of the hydrolysis of phytic acid and lower inositol phosphates

The ITP results depicted in Figs. 1 and 2 allow to observe the time dependence of the formation and decay of all solutes formed during the hydrolysis. The result of the quantitation of the particular compounds is shown in Fig. 3 (where the concentration of the solutes is not given in absolute quantities, e.g. in *M* or in g/l, but as a relative content). This is done such that the length of the ITP zone of the particular component (which is proportional to the amount in the 20-μl sample volume injected) is related to its maximum zone length observed during the entire time of hydrolysis. This depiction is appropriate for the goal of this investigation. A further

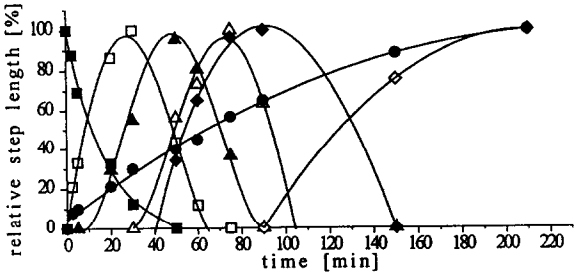

Fig. 3. Time course of the decay and formation of the inositol phosphates during the enzymatic hydrolysis of phytic acid, measured by ITP. ■ = IP6; □ = IP5; ▲ = IP4; △ = IP3; ◆ = IP2; ◇ = IP1; ● = P. For details see text.

quantitation, e.g. by calibration with an external standard is possible. It is not obvious for the case under investigation because the decomposition products are hardly available as standards for calibration. In this case an approach could be carried out by calculating the effective mobilities from the step heights in the isotachopherograms and approximating the concentrations by the Kohlrausch "regulation function". Even an overall mass balance could be made possible in this way. This is, however, not of interest for the problem presented here.

From Fig. 3 it can be seen that half of the initially present IP6 decays within 12 min under the given conditions, whereby the immediate formation of IP5 and P is clearly observed from the appearance of their ITP zones. The concentration of IP5 and P increases upon degradation of IP6. That of IP5 increases steeply, reaches a maximum value after 30 min, and decreases further. The gradual formation of the next reaction product, IP4, is observed after about 10 min. This product shows a nearly symmetric concentration vs. time curve of a similar shape as IP5 (in contrast to IP6, which obviously exhibits an overall decline). The curve of IP4 is, however, broader than that of IP5, an effect which is propagated for the subsequently formed components: the curves have similar shapes but become increasingly broader.

Whereas the concentration of the higher inositol phosphates decreases after the maximum this is not longer the case for IP1: within the reaction time a maximum is approached but no decline is observed. A similar effect is found for P, the concentration of which is continuously increasing directly after the start of the hydrolysis, approaching a maximum value. The latter effect indicates a certain saturation of the hydrolysis due to the fact that the enzyme is probably not capable to hydrolyse IP1 [1].

From these results it can be concluded that ITP serves as a rapid and simple method to determine the time course of the hydrolysis of phytic acid.

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