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Determination of inositol phosphates in fermentation broth using capillary zone electrophoresis with indirect UV detection

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Abstract

The potential of capillary zone electrophoresis for the fast monitoring of a fermentation process in which inositol phosphates are enzymatically hydrolyzed has been investigated. The developed analysis consists of capillary zone electrophoresis combined with indirect UV detection, using 1-naphthol-3,6-disulfonic acid as the chromophore. The total analysis of all six inositol phosphates covering a concentration range of 0–500 μM takes only 13 min.

1. Introduction

Specific inositol phosphates, e.g. 1,4,5-inositol trisphosphate, play an important role as a second messenger in signal transduction in the body [1]. Others, such as phytic acid, are found in grains and seeds [2], whereas specific isomers of inositol trisphosphate show several interesting pharmacological properties [3]. Because of their physicochemical characteristics, fast analysis of inositol phosphates has been a problem for many years. First, inositol phosphates are, depending on the number of phosphate groups, multiply negatively charged, even at low pH values. Second, because of the absence of chromophoric or fluorophoric groups in the molecule sensitive detection is rather complicated. For the separation of the compounds ion-pair [4,5] and ion-exchange chromatography [6,7] have been applied, as well as gas chromatography after derivatization of myo-inositol formed after enzymatic hydrolysis [8]. However, these separation methods are rather time consuming, caused by the equilibration times in ion chromatography or by the need of laborious derivatization procedures in the case of gas chromatography. Detection methods applied include refractive index detection [9], radiometric detection [10], colorimetric detection [11], fluorometry after complexation [3] and after derivatization [12], mass spectrometry [13], post-column reaction detection, based on enzymatic hydrolysis of the phosphate esters and detection of the inorganic phosphate formed [14], electrochemical detection of NADH after enzymatic oxidation of inositol [15] and suppressed conductivity [16].

Since for the monitoring of the enzymatic hydrolysis of phytic acid a fast analysis of charged compounds is required, capillary zone electrophoresis (CZE) enabling indirect UV detection should be an attractive technique. Henshall et al. [17] already described this meth-

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od for IP_1 (1- and 2-isomer), IP_2, IP_3 and IP_6. However, IP_4 and IP_5 were not included and for a routine analysis the method was said to be questionable because of the day-to-day variation in migration time. Capillary zone electrophoresis, which is based on the charge and size of the molecules, appeared to be appropriate for the fast separation of the multiply charged inositol phosphates. It reduces the long analysis times as obtained with chromatographic systems while the loss of material due to adsorption onto the chromatographic support material is avoided.

Indirect detection methods [18] based on either UV, fluorescence or amperometric detection offer the advantage that no derivatization of the compounds is needed. Either a chromophore [19–21], a fluorophore [22–24] or an electrochemically active substance [25] is added to the buffer thus creating a constant, large background signal. Similar mobilities of the buffer constituent and the analyte are of major importance for the resulting peak shapes. The analyte signal is derived from the signal of the buffer constituent through displacement of the electrolyte by the analyte. A severe disadvantage of indirect detection is the increased noise level by the addition of the chromophore with its detection characteristics which leads to increased detection limits.

The present paper describes the determination of myo-inositol phosphates in fermentation broth with CZE and indirect UV detection using 1-naphthol-3,6-disulfonic acid as chromophore. The only clean-up step required consisted of centrifugation of the fermentation sample, the supernatant being directly injected in the CZE system.

2. Experimental

2.1. Chemicals

1-Naphthol-3,6-disulfonic acid (NDSA) was obtained from Janssen (Beerse, Belgium). Acetic acid p.a. was purchased from Baker (Deventer, Netherlands). Both inositol monophosphate (2-IP_1), as dicyclohexylammonium salt and hydroxypropylmethylcellulose (HPMC), with a viscosity of 4000 cP for a 2% aqueous HPMC solution, came from Sigma (St. Louis, MO, USA). Inositol bis- (1,2-IP_2), tris- (1,2,6-IP_3), tetrakis- (1,2,5,6-IP_4), pentakis- (1,2,4,5,6-IP_5) and hexakisphosphate (IP_6) were supplied as sodium salts by Perstorp Pharma (Perstorp, Sweden). For the preparation of the stock solutions of analytes and buffer solutions, deionized water was used (Milli-Q system, Millipore, Bedford, MA, USA). Calibration curves were generated by spiked fermentation buffer with different concentrations of inositol phosphates. The buffer solution was filtered through a 0.2-μm Nylon acrodisc syringe filter (Gelman Sciences, Ann Arbor, MI, USA).

2.2. Electrophoresis

The experiments were performed on a P/ACE 2200 system (Beckman, Fullerton, CA, USA), including a liquid thermostated (24°C) capillary and a UV detector. The electrophoresis medium was prepared freshly every day and consisted of 0.5 m*M* NDSA, 30 m*M* acetic acid and 0.01% HPMC to suppress the electroosmotic flow. The applied voltage was −30 kV generating a current of about 10 μA. Detection was performed at 214 nm with a data sampling rate of 5 Hz and a time constant of 0.5 s. For data collection and handling System Gold software, version 7.12 (Beckman) was used. This software did not integrate the large negative peak preceding the IP_6 peak; integration was set to start at the IP_6 peak base. Untreated fused-silica capillaries (75 μm I.D.) from SGE (Ringwood, Victoria, Australia) with a total length of 0.57 m (0.50 m to the detector) were used. New capillaries were rinsed with deionized water and electrophoresis medium, each for 2 min. Before each injection the capillary was rinsed with electrophoresis medium for 2 min. Pressurized injection during 3 s, which corresponded to 34 nl was applied.

2.3. Sample pretreatment

Samples taken from the fermentation broth containing yeast, buffer and inositol phosphates,

were centrifuged for 5 min in an Eppendorf centrifuge 5415 (Eppendorf Geraetebau, Netheler und Hinz GmbH, Hamburg, Germany) at 11 000 rpm (4000 *g*). The supernatant was introduced into the capillary after 1:1 dilution with fermentation buffer.

3. Results and discussion

3.1. Electrophoresis

Inositol phosphates have in general high electrophoretic mobilities due to the multiple charges of the phosphate groups. When electrophoresis takes place at pH values that are favourable regarding selectivity, the electroosmotic flow (EOF) under these conditions (pH about 3) is rather low. At higher pH values, the EOF increases considerably, while simultaneously the selectivity between IP_4, IP_5 and IP_6 becomes unacceptably low. As a consequence, the peaks are not sufficiently separated anymore to quantitate all peaks. Because the phosphate groups are negatively charged, this implies that without electroosmotic flow the analytes migrate in the direction of the anode. Since the electrophoretic velocity is the resultant of the electrophoretic mobility and the electroosmotic mobility the net result at the applied pH is rather low electrophoretic velocities leading to unacceptably long analysis times. Therefore, it has been decided to reverse the polarity of the system (negative inlet electrode, grounded outlet electrode). In that case it is necessary to suppress the electroosmotic flow as much as possible, which is realized by modifying the electrophoresis buffer. Although the stability of dynamically coated capillary walls are not as favourable as untreated capillaries, we obtained quite acceptable systems using HPMC which is demonstrated by the validation figures.

Due to the many phosphate groups of the inositol phosphates, the pH of the electrophoresis medium is a very critical parameter in the separation of inositol phosphates: 0.2 pH units deviation already induced considerable changes in the migration time. Thirty millimolar of acetic acid (pH 3.0) appeared to be adequate for this purpose. Migration times and relative standard deviations (R.S.D.) of all six inositol phosphates are shown in Table 1. These are mean values from 15 measurements over the whole concentration range. The R.S.D. for all the compounds is less than 2.6% allowing reliable peak identification. Because the effect of electrodispersion is more pronounced at higher concentrations, the migration times of the compounds that show fronting (IP_4, IP_5, IP_6) tend to be slightly higher at increased concentrations, whereas the migration times of the compounds that show tailing (IP_2, IP_1) are reduced at higher concentrations.

3.2. Detection

With respect to indirect detection, NDSA has been chosen as the chromophore because its electrophoretic mobility matches closely with that of the most important analyte, IP_3. The optimal matching has been obtained by adjustment of the pH of the electrophoresis buffer. As a consequence of the high optical background the noise of the baseline is considerably increased in comparison with direct UV detection. Fig. 1 presents the electropherogram of the inositol phosphates having analysis times of less than 6 min. Under these conditions the isomers are not separated. Although IP_5 and IP_6 are not baseline separated, they still can be quantified. The other inositol phosphates are completely baseline separated. From this figure it can easily be seen that IP_4, IP_5 and IP_6 have electrophoretic mobilities higher than that of the chromophore

Table 1
Migration times and relative standard deviation (R.S.D.) of the inositol phosphates

Compound	Migration time (s)	R.S.D. (%)
IP1	315.5	0.4
IP2	240.2	1.6
IP3	205.3	0.9
IP4	187.0	2.0
IP5	176.7	2.5
IP6	166.4	1.4

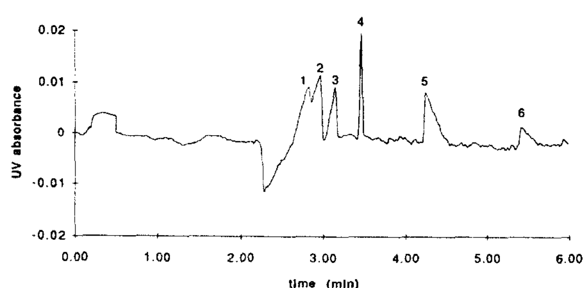

Fig. 1. Electropherogram of a standard mixture of all six inositol phosphates (1 = 340 μM IP_6, 2 = 320 μM IP_5, 3 = 160 μM IP_4, 4 = 170 μM IP_3, 5 = 220 μM IP_2, 6 = 500 μM IP_1). Conditions: applied voltage: −30 kV, current: 10 μA, λ = 214 nm, fused-silica capillary: 75 μm I.D., electrophoresis buffer: 0.5 mM NDSA, 30 mM acetic acid, 0.01% HPMC.

at pH 3, which leads to fronting peak shapes. On the other hand, IP_1 and IP_2 have lower electrophoretic mobilities resulting in tailing peaks. This asymmetry of the peaks is caused by electrodispersion, deriving from local differences in conductivity and consequently differences in the local electric field strengths. This effect is more pronounced at concentrations of the analytes that are high in comparison with the chromophore [21]. IP_3 is the only compound with a symmetrical peak shape. By adjusting the mobility of the chromophore to that of the analyte, electrodispersion of that particular compound is suppressed and the efficiency is improved [20]. In that way the limit of determination (LOD) of the analyte can be improved. Therefore, the LOD (defined as 10 times the noise) appeared to be lowest for IP_3 (3.9 μM). The LODs for IP_4 and IP_6 amounted to 9.2 and 22.5 μM, respectively, applying an injection volume of 34 nl.

In a fermentation process, however, the concentrations of the main compounds of interest are in the 50–1000 μM range. For that reason the LOD of the compounds is not critical.

3.3. Quantitative aspects

Quantitative aspects have been examined by generating calibration curves for the compounds of interest. Therefore, fermentation buffer was spiked with concentrations of inositol phosphates up to 1 mM. Calibration curves for the inositol phosphates are linear in the 0–500 μM range. For concentrations above 500 μM being the concentration of the chromophore the peak area of the inositol phosphates does not increase linearly. By increasing the chromophore concentration, higher inositol phosphate concentrations can be determined. Unfortunately, the linear dynamic range only shifts to higher concentrations but is not expanded, while the LOD is still increased. Calibration plots for the inositol phosphates were thus made from 0–500 μM. The calibration plots of IP_1 and IP_6 have the lowest correlation coefficients of 0.993 and 0.986, respectively, which is caused by the least symmetric peak shapes. The correlation coefficients for the other inositol phosphates were higher: 0.998 (IP_2), 0.996 (IP_3), 0.997 (IP_4) and 0.995 (IP_5).

The developed analysis has been validated for the most important analytes in the fermentation mixture, IP_2, IP_3, IP_4 and IP_6. The intra-day and inter-day variability, expressed as imprecision (R.S.D., %), have been examined for different concentrations of inositol phosphates (Table 2). The intra-day variability did not exceed 19.8%

Table 2
Intra-day and inter-day variability expressed as imprecision (R.S.D.) of the method

Compound	Concentration (μM)	Intra-day		Inter-day R.S.D. (%), n = 3
		R.S.D. (%)	n	
IP_2	348	9.6	11	1.2
		14.5	8	
IP_3	99	4.4	20	2.4
		5.7	25	
		6.1	16	
	395	3.9	6	2.5
		8.9	8	
IP_4	192	13.6	20	1.4
		10.0	25	
		8.1	17	
	383	9.0	6	1.2
		12.7	8	
IP_6	396	19.8	20	7.8
		19.8	26	
		13.1	19	

(IP_6), whereas the highest inter-day variability amounted to 7.8% (IP_6). As can be seen from Table 2, the developed method shows a good reproducibility. Although the intra-day variability for IP_6 is relatively high, its value is acceptable for this application. Nevertheless, it indicates that every day a new calibration curve for the different analytes has to be constructed. The analytes for the calibration curves are dissolved in the fermentation buffer in order to simulate the real samples as much as possible thus increasing the accuracy of the analysis. Fermentation samples are diluted 1:1 with the fermentation buffer to avoid concentrations out of the linear range.

3.4. Fermentation monitoring

Fermentation monitoring, being the aim of the analysis, has been performed after a simple sample pretreatment of centrifugation which takes only 5 min. During the centrifugation the yeast is separated as a pellet from the inositol phosphates in the supernatant. Fig. 2 shows the electropherograms of the fermentation broth analyzed after 5 min (Fig. 2a), 60 min (Fig. 2b) and 22 h (Fig. 2c). From these electropherograms it is evident that the applied sample pretreatment, though in the off-line mode, is sufficient for quantification of the analytes. IP_6, present at a high initial concentration (Fig. 2a), has been hydrolyzed into the other inositol phosphates and free phosphate after a few hours (Fig. 2b and 2c). The potential of monitoring the fermentation process is also demonstrated in Fig. 3. The more active the yeast, the faster phytic acid is hydrolyzed. During the hydrolysis a high amount of free phosphate has been formed. Phosphate has approximately the same electrophoretic mobility as IP_1. For that reason IP_1 can not be determined in the fermentation broth. At even higher phosphate concentrations, interference with IP_2 also occurs. A minor drawback of the developed analysis is the off-line sample pretreatment. Instead of centrifugation which cannot easily be automated, dialysis but especially electrodialysis has to be explored as sample pretreatment. If a dialysis probe is positioned in the fermentation broth, a connection between the dialysis probe and a sample vial will allow the complete automation of the analytical method.

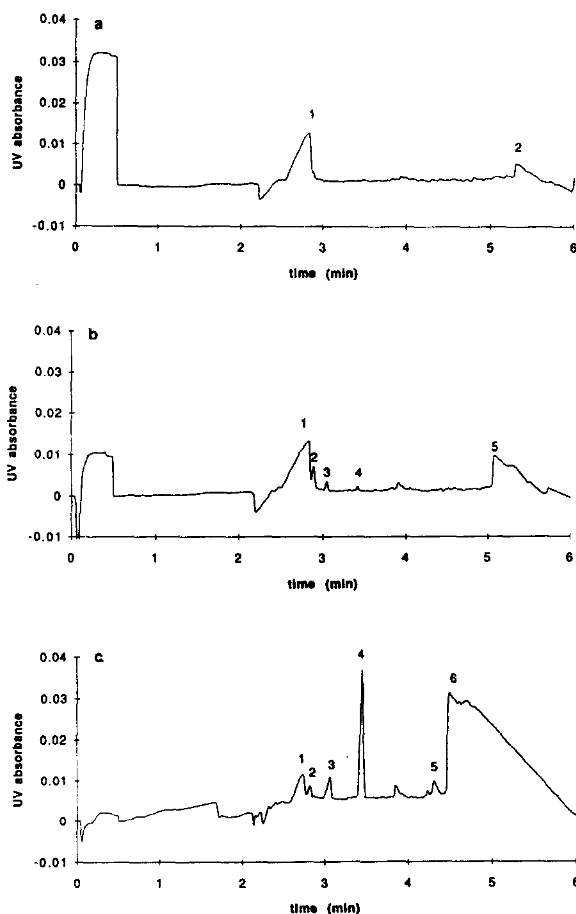

Fig. 2. Electropherograms of a sample of the fermentation broth at 5 min (a), 60 min (b) and 22 h (c). Concentrations of the inositol phosphates: (a) 1 = 480 μM IP_6, 2 = acetate, (b) 1 = 420 μM IP_6, 2 = 30 μM IP_5, 3 = 20 μM IP_4, 4 = 8 μM IP_3, 5 = phosphate + acetate, (c) 1 = 70 μM IP_6, 2 = 20 μM IP_5, 3 = 60 μM IP_4, 4 = 300 μM IP_3, 5 = 30 μM IP_2, 6 = phosphate + acetate. For conditions see Fig. 1.

4. Conclusions

A method for monitoring the enzymatic hydrolysis of phytic acid has been developed. Fast separation and detection of all six inositol phosphates in deionized water has been achieved by using capillary zone electrophoresis with indirect

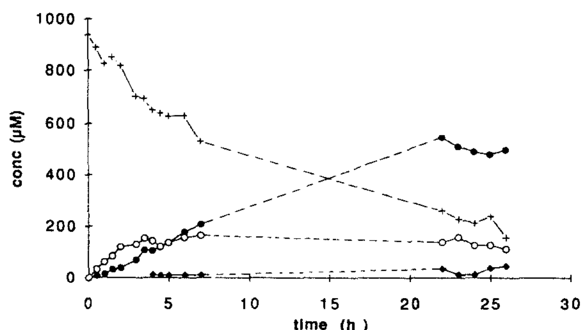

Fig. 3. Monitoring of the enzymatic hydrolysis of phytic acid. ◆ = IP_2, ● = IP_3, ○ = IP_4, + = IP_6.

UV detection. The mobility of the chromophore has to be adjusted to that of the compound to be quantified most accurately.

In the fermentation broth the high concentration of free phosphate masks IP_1 thus preventing it from being detected. At long reaction times the high concentration of free phosphate formed during fermentation may also interfere with the detection of IP_2.

References

[1] N.N. Osborne, A.B. Tobin and H. Ghazi, *Neurochem. Res.*, 13 (1988) 177.
[2] E. Graf, *J. Agric. Food Chem.*, 31 (1983) 851.
[3] H. Irth, M. Lamoree, G.J. de Jong, U.A.Th. Brinkman, R.W. Frei, R.A. Kornfeldt and L. Persson, *J. Chromatogr.*, 499 (1990) 617.
[4] J.A. Shayman and D.M. BeMent, *Biochem. Biophys. Res. Comm.*, 151 (1988) 114.
[5] A.S. Sandberg and R.J. Ahderinne, *J. Food Sci.*, 51 (1986) 547.
[6] R. Ellis and E.R. Morris, *Cereal Chem.*, 59 (1982) 232.
[7] R.E. Smith and R.A. Macquarrie, *LC-GC*, 7 (1989) 775.
[8] C.W. Ford, *J. Chromatogr.*, 333 (1985) 167.
[9] B. Tangendjaja, K.A. Buckle and M.J. Wootton, *J. Chromatogr.*, 197 (1980) 274.
[10] H. Binder, P.C. Weber and W. Siess, *Anal. Biochem.*, 148 (1985) 220.
[11] J.J.L. Cilliers and P.J. v. Niekerk, *J. Agric. Food Chem.*, 34 (1986) 680.
[12] M.E. Kargacin, G. Bassel, P.J. Ryan and T.W. Honeyman, *J. Chromatogr.*, 393 (1987) 454.
[13] W.R. Sherman, K.E. Ackerman, R.A. Berger, B.G. Gish and M. Zinbo, *Biomed. Environ. Mass Spectrom.*, 13 (1986) 333.
[14] J. Meek, *Natl. Acad. Sci.*, 83 (1986) 4162.
[15] G. Marko-Varga, E. Dominguez, B. Hahn-Hägerdal and L. Gorton, *J. Pharm. Biomed. Anal.*, 8 (1990) 817.
[16] *Dionex application note AN 65*, 1990, Dionex, Sunnyvale, CA.
[17] A. Henshall, M.P. Harrold and J.M.Y. Tso, *J. Chromatogr.*, 608 (1992) 413.
[18] E.S. Yeung and W.G. Kuhr, *Anal. Chem.*, 63 (1991) 275A.
[19] S. Hjerten, K. Elenbring, F. Kilar, J. Liao, A.J.C. Chen, C.J. Siebert and M. Zhu, *J. Chromatogr.*, 403 (1987) 47.
[20] F. Foret, S. Fanali, L. Ossicini and P. Bocek, *J. Chomatogr.*, 470 (1989) 299.
[21] G.J.M. Bruin, A.C. v. Asten, X. Xu and H. Poppe, *J. Chromatogr.*, 608 (1992) 97.
[22] W.G. Kuhr and E.S. Yeung, *Anal. Chem.*, 60 (1988) 1832.
[23] T.W. Garner and E.S. Yeung, *J. Chromatogr.*, 515 (1990) 639.
[24] M.D. Richmond and E.S. Yeung, *Anal. Biochem.*, 210 (1993) 245.
[25] T.M. Olefirowicz and A.G. Ewing, *J. Chromatogr.*, 499 (1990) 713.