Annals. Food Science and Technology
2009

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC MONITORING OF ENZYMATIC STARCH HYDROLYSIS

E. Muntean
University of Agricultural Sciences and Veterinary Medicine, Chemistry Department,
3 – 5 Calea Mănăştur, 400383- Cluj Napoca, Romania
E-mail: edimuntean@yahoo.com

Abstract
Enzymatic starch hydrolysis is one of the most important processes in industry, as the production of many sweeteners, pharmaceuticals and animal feeds needs low molecular mass compounds obtained from starch. Starch hydrolysis to glucose being never complete, usually leads to a mixture of glucose, maltose and oligosaccharides; a proper control of the starch enzymatic hydrolysis is essential in order to obtain products with desired properties such as viscosity or sweetening power. As classical monitoring methods are time-consuming, inaccurate, involving also extensive sample handling, the purpose of this research was to surpass these disadvantages and to develop an appropriate analytical method, using high performance liquid chromatography (HPLC), with the following restrictions: performing separations on commercially available column and mobile phase, a minimum sample workup and a reasonable analysis time, minimizing thus the analysis costs. This paper reports a HPLC method using differential refractive index detection for monitoring starch hydrolysis; isocratic separations were carried out on a Nucleodur 100 – 5 NH_2 RP column, using as mobile phase a mixture of acetonitrile : water, the total run time being less than 20 minutes. The method has been validated for specificity, linearity, detection limit, recovery, accuracy and precision for fructose, glucose, sucrose and maltose; high correlation coefficients (>0.995) were obtained for calibrations of all four carbohydrates, the method proving to be selective, accurate (recoveries between 95–102%) and precise (<5% relative standard deviation). The proposed method is remarkable simple and fast, being successfully applied in monitoring lab-scale starch hydrolysis using commercial enzymes. HPLC analysis of liquefied starch revealed only a small amount of glucose, while during monitoring the saccharification stage, which was performed with different concentration of Dextrozyme, HPLC analysis revealed that in the early stage of hydrolysis, Dextrozyme produced besides glucose, small amounts of fructose, while using diluted Dextrozyme fructose is missing, besides glucose a small amount of maltose being detectable.

Keywords: HPLC, starch, hydrolysis, analysis, monitoring, carbohydrates, glucose, fructose, maltose

1. INTRODUCTION

Starch hydrolysis is a major industrial enzymatic bioconversion and one of the most important processes in food industry, as the production of many natural sweeteners, pharmaceuticals, textiles and animal feeds needs low molecular mass compounds obtained from starch [9]. Acid hydrolysis of starch has had widespread use in the past, but it required the use of corrosion resistant materials, gave rise to high color and salt content, needed more energy for heating and was relatively difficult to control [10].
Nowadays, hydrolysis is usually carried out using enzymes, these ensuring a higher reaction rate while requiring lower temperatures, normal pressure and mild pH values [4, 12]. The process takes place in three consecutive steps: gelatinization, liquefaction and saccharification. Gelatinization opens the starch granules to hydration and enzymatic hydrolysis by breaking of hydrogen bonds among and within starch molecules, being usually accomplished by heating in water, at 90 - 100°C. During liquefaction, an α-amylase cleaves 1,4-glycosidic bonds to yield shorter dextrins chains; in the last saccharification stage, a glucoamylase attacks 1,4-terminal bonds of dextrins to release glucose. The values of optimum pH, temperature and other operating conditions differ between the enzymatic stages; enzymatic hydrolysis can be stopped at dextrins or at glucose, depending which is the final product. Alternatively, isomerisation can complete this three stage sequence, converting glucose to fructose, which posses a higher sweetening power.
The starch hydrolysis to glucose being never complete, usually leads to a mixture of glucose, maltose and oligosaccharides; a proper control of the enzymatic process is essential in order to obtain products with the desired properties such

as viscosity or sweetening power. Usually, such a control is accomplished by determining the reducing sugar content expressed as dextrose equivalent using the Luff–Schoorl titrimetric method or the Lane Eynon method [14].
However, these methods are time-consuming, inaccurate and involve extensive sample handling. A much convenient control of the process can be achieved by using HPLC for the determination of individual carbohydrates [11]. Gel permeation [3, 13], reverse-phase [6] and silica [5] columns have been used for HPLC analysis of carbohydrates, but the most popular are amino-bonded silica [8] and anion-exchange columns [1, 2].
The purpose of this research was to develop an HPLC method appropriate for analysis of starch enzymatic hydrolysis products, using commercially available column and mobile phase, a minimum sample workup and a reasonable analysis time, minimizing thus the analysis costs.

2. MATERIALS AND METHODS

Starch, glucose, fructose, saccharose and maltose were all reagent-grades (Merck); HPLC grade acetonitrile (Merck) was used for mobile phase preparation, together with ultrapure water produced with a Millipore Direct Q 3UV Smart. Enzymatic hydrolysis was achieved with Termamyl® 2X and Dextrozyme® GA (both from Novozymes A/S Denmark). Buffer solution with pH 4.5 was prepared from reagent grade KH_2PO_4 and $K_2HPO_4.3H_2O$ (Merck).
Sample workup is fast and simple, consisting in dilution followed by filtration through 0.45 μ membrane filter (Millipore).
HPLC analyses were performed on a Shimadzu system, consisting in a Prominence DGU 20As degasser, a Prominence LC-20AD solvent delivery module, an automatic sample injector SIL-10AF, a RID-10A differential refractive index detector, a Prominence CTO-20A column oven and a system controller Prominence CBM-20Lite. Instrument control, data acquisition and data analysis were accomplished by „LCsolution" ver.1.2. software. Isocratic separations were carried out on a EC 250/ 4 Nucleodur 100–5 NH_2 RP column using as mobile phase 77% v/v acetonitrile in water, at 1 mL/ min and 35^0C, with an injection volume of 10 μL. The separation parameters were established by optimization using a 2^3 factorial design, the tested experimental domain being characterized by the following limits: 30 – 40^0C for temperature, 0.8 – 1.4 mL/ min for flow rate and 20 – 30% water in the mobile phase [7], leading to a total separation time less than 23 minutes (fig.1).

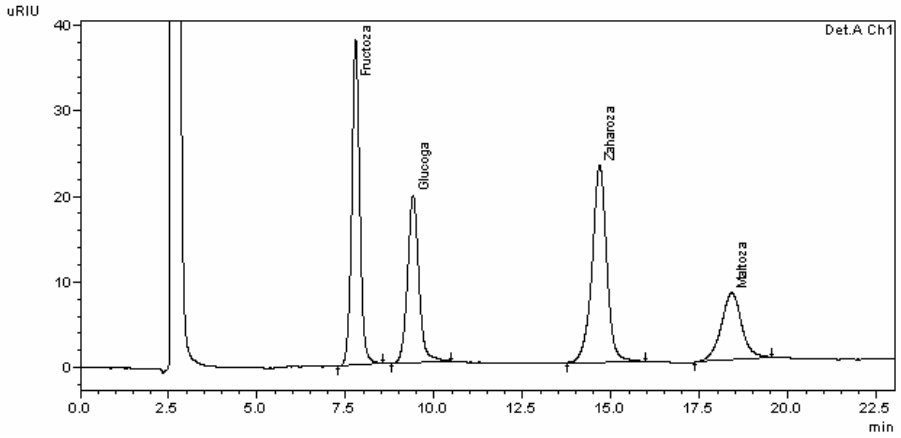

Figure 1. HPLC chromatogram of a reference mixture of carbohydrates

Starch enzymatic hydrolysis followed the gelatinization, liquefaction and saccharification stages: 5 g starch was homogenized in 25 mL cold water, then the mixture was homogenized again after adding 75 mL hot water. The obtained gel was subjected to liquefaction, after

adjusting the pH to 6 with phosphate buffer and heating to 105^0C; 5 mL Termamyl were then added and the reaction mixture was kept on a thermostated stirrer (Heidolph MR Hei-Standard) for 20 minutes at 105^0C.For saccharification, the pH of 100 mL liquefied starch aliquots were adjusted to 4.5, the reaction vessel was thermostated at 60^0C and 500 µL Dextrozyme were added. At intervals of 10, 30, 60, 90 and 120 min, 10 mL aliquots were withdrawn from the reaction mixture to determine carbohydrates using HPLC; for stopping the enzymatic reaction, 1 mL of 0.1 M HCl was added before each determination.

3. RESULTS AND DISCUSSION

Quantitative analyses were performed using the external standard method; calibrations were achieved using five levels of concentration, for accurately determine the concentration of target analytes. The calibration curves show a good linearity with R > 0.998 as indicated in table 1.

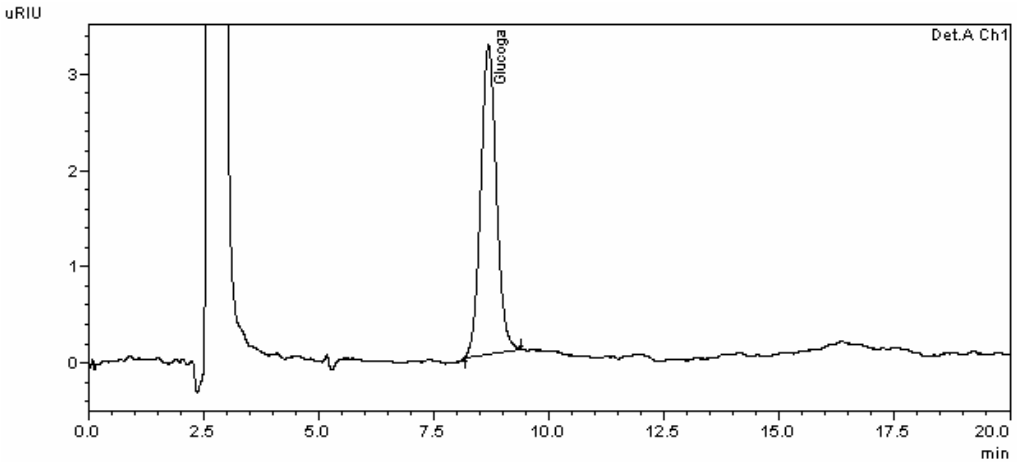

Figure 2: HPLC chromatogram of the liquefaction product obtained with Termamyl

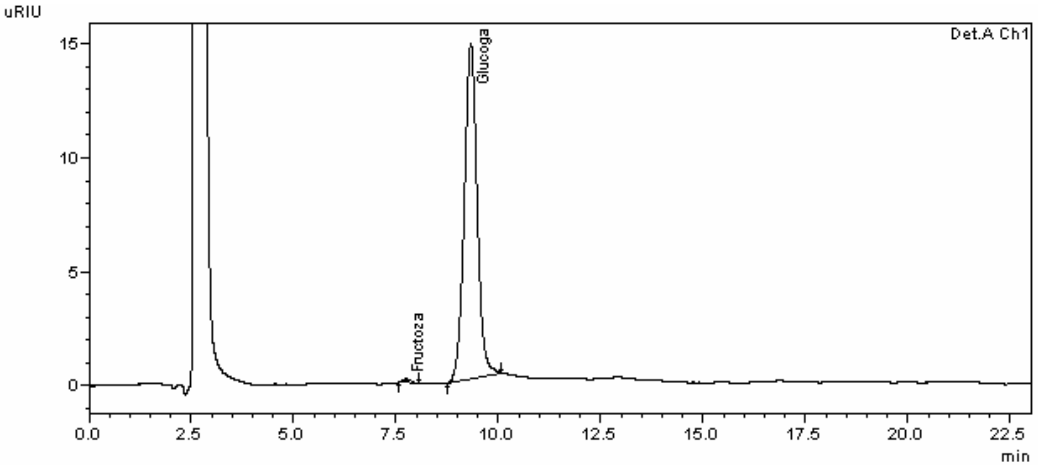

Figure 3: HPLC chromatogram of the hydrolysate obtained with commercial Dextrozyme

As the starting point of saccharification is liquefaction, a first target of HPLC analysis was the liquefied starch; only a small amount of glucose was detected (figure 2).Saccharification was performed with two concentrations of Dextrozyme, using this enzyme as it is commercially available in a first case, then diluted 1/ 100. Two different reaction

mechanisms were revealed, as a function of enzyme/ substrate concentrations ratio. For the case of undiluted Dextrozyme, small amounts of glucose were converted to fructose, as observed from figure 3, the maximum level of fructose being observed in the early stage of hydrolysis, after ten minutes a constant decrease being recorded (figure 5).
The diluted Dextrozyme produced small amounts of maltose besides glucose, but these were generated only starting with the minute 30 of reaction (figure 6), while fructose is missing

Table 1: Results of regression analysis for calibrations

Analyte	Regression equation*	R^2	Quantification limits [g/ L] vs. linear range
Fructose	C = 0.0132 . A – 76.1499	0.9985	0.102 0.467 ... 7.778
Glucose	C = 0.0175. A + 0.6077	0.9987	0.154 0.453 ... 7.545
Saccharose	C = 0.0124 . A + 53.7045	0.9993	0.112 0.496 ... 8.265
Maltose	C = 0.0228 . A + 88.7727	0.9987	0.115 0.436 ... 7.272

*C: concentrations [g/ L]; A: peak areas

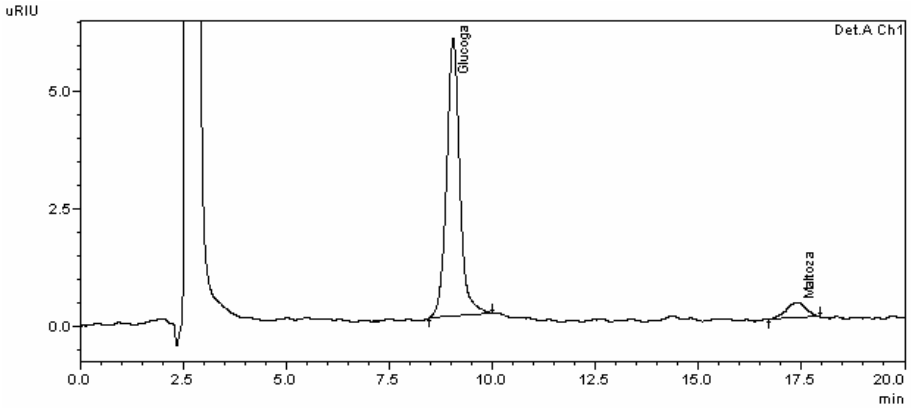

Figure 4: HPLC chromatogram of the hydrolysate obtained with diluted Dextrozyme

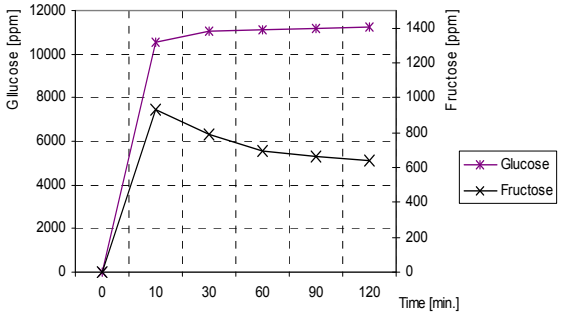

Figure 5: Concentration profiles for the reaction's products obtained with Dextrozyme

4. CONCLUSIONS

A simple and rapid HPLC method involving differential refractive index detection and a minimal sample preparation (dilution followed by filtration) was developed for monitoring enzymatic starch hydrolysis. The proposed method is remarkable simple and fast, being successfully applied in monitoring lab-scale starch hydrolysis using commercial enzymes.

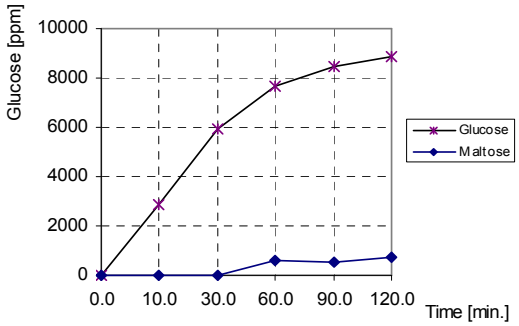

Figure 6: Concentration profiles for the reaction's products obtained with diluted Dextrozyme

5. ACKNOWLEDGMENTS

This research was conducted in the framework of the National Partnership research project nr.51001/ 2007.

6. REFERENCES

[1] Andersen R., Sørensen A., Separation and determination of alditols and sugars by high-pH anion-exchange chromatography with pulsed amperometric detection. Journal of Chromatography A, 2000, 897, 195-204.

[2] Cataldi T.R.I., Angelotti M., Bianco G., Determination of mono- and disaccharides in milk and milk products by high-performance anion exchange chromatography with pulsed amperometric detection. Analytica Chimica Acta, 2003, 485, 43-49.

[3] Chen Z.L., Hibbert D.B., Simultaneous amperometric and potentiometric detection of sugars, polyols and carboxylic acids in flow systems using copper wire electrodes. Journal of Chromatography A, 1997, 766, 27-33.

[4] Haki G.D., Rakshit S.K., Developments in industrially important thermostable enzymes: a review. Bioresource Technology, 2003, 89, 17-34.

[5] Linden J.C., Lawhead C.L., Liquid chromatography of saccharides. Journal of Chromatography, 1975, 105, 125-133.

[6] McGinnis G.D., Prince S., Lowrimore J., The use of reverse-phase columns for separation of unsubstituted carbohydrates. Journal of Carbohydrate Chemistry, 1986, 5, 83-97.

[7] Muntean E., Muntean N., Optimization of the carbohydrates' High Performance Liquid Chromatographic analysis conditions. In: Proceedings of The 33rd Annual ARA Congress, Sibiu, Romania, 2009

[8] Nogueira L.C., Silva F., Ferreira I.M.P.L.V.O., Trugo L.C., Separation and quantification of beer carbohydrates by high-performance liquid chromatography with evaporative light scattering detection. Journal of Chromatography A, 2005, 1065 (18) 207-210.

[9] Othmer K., Kirk-Othmer Encyclopedia of Chemical Technology. Wiley Interscience, 10, 248 – 317, 2005.

[10] Palmer T.J., Glucose Syrups and related carbohydrates. Birch, G.G., Green, L.F., Coulson. C.B., eds., Applied Science, London, 1970.

[11] Robards K., Whitelaw M., Chromatography of monosaccharides and disaccharides, Journal of Chromatography, 1986, 373, 81-110.

[12] Van der Maarel M., Bart van der Veen J.E.C., Uitdehaag J.C.M., Leemhuis H., Dijkhuizen L., Properties and applications of starch- converting enzymes of the α-amylase family. Journal of Biotechnology, 2002, 94, 137-155.

[13] Whistler R.L., Anisuzzaman A.K.M., Gel Permeation Chromatography. Methods in Carbohydrates Chemistry, 1980, 8, 45-49.

[14] International Standards Organization. Starch hydrolysis products - Determination of reducing power and dextrose equivalent - Lane and Eynon constant titration method. ISO 5377-1981, Geneva, Switzerland, 1981.