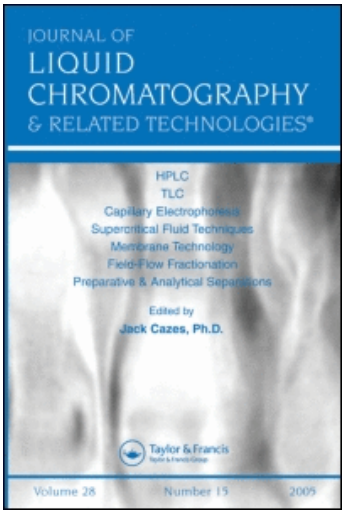

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Biochemistry Reaction and Separation in a RP-HPLC System for Starch Hydrolysis

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Biochemistry Reaction and Separation in a RP-HPLC System for Starch Hydrolysis

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Abstract: The combination of a biochemical reaction and a chromatographic separation was successfully performed for starch hydrolysis catalyzed by α-amylase, using a RPLC system in a preparative size column. The mobile phases were solutions of α-amylase at controlled concentrations. The substrate was starch. The results showed that the reaction product was dextrin hydrate, the intermediate product of starch hydrolysis. The experimental conditions for the separation and the reaction were studied to investigate their individual influence on the reaction chromatography process.

Keywords: Reaction chromatography, α-Amylase, Starch, Hydrolysis, Reverse phase high performance liquid chromatography, Preparative chromatography

INTRODUCTION

Reaction chromatography is gaining more attention as a hybrid process, which allows reaction and separation to be conducted in a single step. Since the reaction product is generated and separated from the reagents at the same time, within the column, it is possible to obtain the object product fraction by selecting suitable experimental conditions. Biochemistry products, e.g., polysaccharides, proteins, and amino acids can often be separated by chromatography. Usually these products come from enzyme catalyzed reactions

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such as hydrolysis. In these cases, reaction chromatography offers the potential to simultaneously prepare and separate these biochemicals.[1–3] The advantage of a technology coupling reaction and chromatography is that it can increase the production rate and lessen the amount of labor needed compared with the conventional biochemistry reaction process.

Reaction chromatography was first applied to the research of reaction kinetics and the characteristics of some catalysts by gas reaction chromatography (GCR).[4,5] Comprehensive development of theory and experimental investigation with GCR has facilitated understanding and development of liquid chromatographic reactors (LCR), particularly with regard to the potential of more widespread use of HPLC. With the development of chromatography, some technologies, such as continuous annular chromatography (CAC) and simulated moving bed (SMB) make the process of chromatography continuous.[6–8]

Though reaction chromatography can take place continuously, initial work generally involves batch chromatography. Batch chromatography also provides a laboratory scale method to prepare reagents.

The hydrolysis of starch into lower molecular weight products, catalyzed by α-amylase, is one of the most important commercial enzyme processes. The hydrolysis products are widely used in foods, as natural sweeteners, and in the production of pharmaceuticals and detergents.[9,10] This paper reports on the reaction of starch hydrolysis carried out in a RPLC system. The products of this reaction chromatography process are only dextrin hydrates. The influence of the two kinds of experimental parameters, those characterizing the separation conditions and the reaction conditions that affect the reaction chromatography process, were studied and optimized.

EXPERIMENTAL

The catalyst was α-amylase (enzyme activity, 20000 unit/g, from the Anshan Beer Company, Anshan, China), soluble starch and maltose (BR grade, from the Beijing OBX Biotechnique Ltd, Beijing, China), dextrin hydrate (BR grade, from the Shen Yang Chemical Company, ShenYang, China), glucose (BR grade, from the Shanghai National Pharmacy Company, Shanghai, China), acetonitrile (chromatography grade, from the Shenyang Chemical Company, ShenYang, China). The water was prepared with a Wa Haha device obtained from Hangzhou WaHaha Purification Water Ltd., (Hanhzhou, China).

The experiments were carried out using a SPD-10Avp Shimazu HPLC instrument (Kyoto, Japan), equipped with a UV and a RI detector. The RI detector response (at a range of 16) was recorded using the HPLC computer data acquisition system, at a rate of 4 Hz. The reaction products were analyzed on a 4.6 $\times$ 150 mm, 5 μm particles, amido column from Agilent

Technology (Palo Alto, CA, USA). The preparative columns were home packed with spherical ODS particles from GEAgel, Beijing, China.

The preparative columns were made of two 10 × 2 cm and two 1 × 2 cm columns packed with 20 and 50 μm spherical ODS, respectively. The columns were dry packed. The mass of ODS in the columns was recorded. Initially a solution of methanol and water (50:50, v/v) was pumped through the column at flow rates ranging between 0.4 mL/min and 4.0 mL/min incremented by 0.4 mL/min to remove residual gas following dry packing and to help the ODS particles to distribute more uniformly within the column.

A series of 1%, 1.5%, and 2% (w/v) starch solution were prepared using boiling water. A series of 0.24%, 0.32%, and 0.48% (v/v) enzyme solution were prepared using purified water.

RESULTS AND DISCUSSION

Process of HPLC Reaction Chromatography

Concentrations of α-amylase solution (0.24%–0.48%, v/v) were pumped into the preparative column until a stable constant baseline was reached. Then, 20 μL aliquots of the starch solution (1–2%) were injected *via* a 50 μL injection loop. The results of the reaction chromatography are shown in Figure 1. The chromatograms of the substrate and α-amylase are also shown in Figure 1 for comparative purposes.

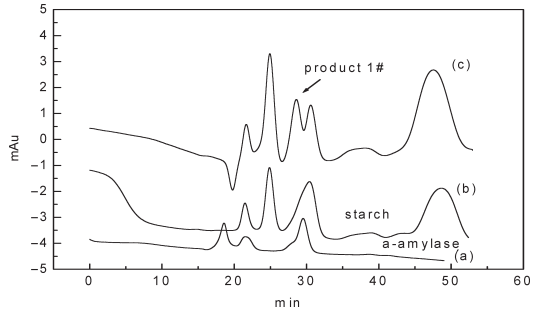

Figure 1. RPLC Chromatograms of the products of starch hydrolysis catalyzed by α-amylase. (a) Chromatogram of a 0.32% (v/v) α-amylase solution; mobile phase water, 50°C. (b) Chromatogram of a 2.0% (w/v) starch solution; mobile phase water, 50°C. (c) Chromatogram of the products of reaction chromatography. Experimental conditions: column 10 × 2 cm, packed with 20 μm ODS-silica; mobile phase, 0.32% (v/v) α-amylase; sample, 2.0% (w/v) starch solution; flow rate, 1.0 mL/min; column temperature 50°C; column efficiency, N = 2317.

Effect of the Separation Conditions in Reaction Chromatography

Effect of the Particle Size and the Column Size in Reaction Chromatography

The performance of the columns were tested using a phenol solution with a mobile phase of methanol:water (85:15, v/v) at a linear flow rate of 1 cm/min. Table 1 summarizes the results. The hold-up time for the columns was determined using uracil. As anticipated, the results shown in Table 1 demonstrate that the column packed with 20 μm C_{18} particles provided better performance.

Four separate columns were used to carry out reaction chromatography experiments. No product #1 was observed except for the 10 × 2 cm i.d. column packed with 20 μm C_{18} silica. This may indicate that the column performance plays an important role in acquiring product #1. Figure 2 shows the elution profiles obtained in reaction chromatography with the columns using different particle and column sizes.

The three separations shown in Figure 2 were also subjected to fraction collection by cutting the eluent around 28 min. The collected fractions were analyzed using the HPLC conditions given in Figure 6. It was determined that no product #1 was present in the fractions collected for chromatograms Figures 2(b) and 2(c). From Figure 2, and the results of analyses for the fraction collections, it is established that reaction chromatography is greatly influenced by column dimensions and particle size. Even in the case of the chromatogram in Figure 2 (a) the reaction rate is small, such that the chromatographic signal obtained for the product is not large. Despite this, Figure 2 (a) demonstrates the importance of chromatographic performance conditions to manufacture and detect product #1.

Effect of the Flow Rate on Reaction Chromatography

A higher flow rate can result in reduced resolution due to mass transfer limitation. Therefore, it is important to optimize the flow rate at a small scale. A fixed injection volume and concentration were used to study the effect of

Table 1. Performance characteristics of four packed columns by dry method (1 cm/min)

Type of packing	10 × 1 cm i.d.		Column		10 × 2 cm i.d.		Column	
	m_i(g)	ε	k′	N	m_i(g)	ε	k′	N
20 μm C_{18}	5.70	0.72	1.68	1645	18.86	0.65	1.36	2317
50 μm C_{18}	4.70	0.71	1.92	328	18.35	0.67	1.33	527

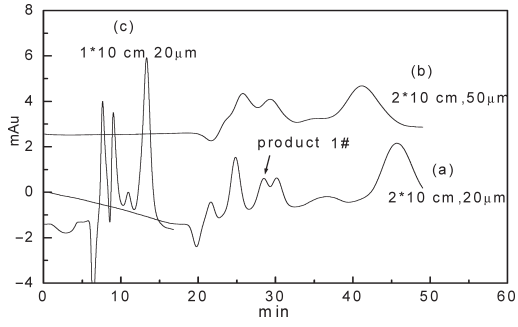

Figure 2. Influences of the average particle size and the column diameter on the process of reaction chromatography. (a) 10 × 2 cm i.d. column packed with 20 μm C_{18} silica, N = 2317, (b) 10 × 2 cm i.d. column packed with 50 μm C_{18} silica, N = 527, (c) 10 × 1 cm i.d. column packed with 20 μm C_{18} silica, N = 1137. Experimental conditions: mobile phase, solution at 0.24% (v/v) α-amylase; sample, 1.0% (w/v) of starch solution; column temperature, 55°C; flow rate, 0.8 mL/min; injected amount, 20 μL.

flow rate. Figure 3 shows the chromatographic profiles obtained when the flow rate was varied from 0.8 to 1.0 mL/min. The lower flow rate shows superior resolution of the product #1. The yield of product #1 at the rate of 0.8 mL/min and 1.0 mL/min was 6% and 2.3%, respectively.

Since the reaction takes place in the mobile phase, the reaction chromatography equations are:

$$u \cdot \frac{\partial C_i}{\partial x} + \frac{\partial C_i}{\partial t} + F\frac{\partial f_i}{\partial t} = uk_r c_1 \tag{1}$$

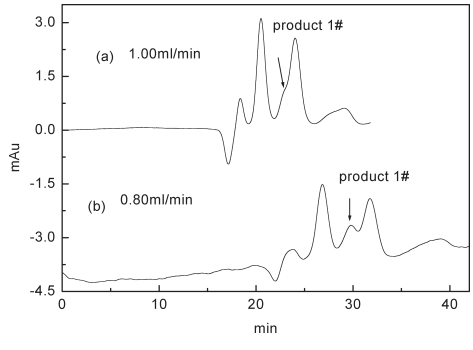

Figure 3. Influence of the flow rate on the reaction chromatography process. Experimental conditions: 10 × 2 cm i.d. column packed with 20 μm ODS silica; mobile phase, 0.24% (v/v) α-amylase solution; sample, 1.0% (w/v) of a starch solution; injected amount, 20 μL; column temperature, 55°C.

where $C_i(I = 1, 2)$ are the concentrations of the starch and the reaction product, respectively, k_r is the rate constant of the reaction, u is the average linear mobile phase velocity, and F is the phase ratio. From this equation, the areas of the products are:

$$A_2 = c_{10}t_p\left[1 - \exp\left(-k_r\frac{L}{u}\right)\right] \tag{2}$$

A detailed solution obtained by numerical calculation has been reported by Lin and Guiochon.[11] The hold-up time is:

$$t_m = \frac{L}{u}$$

And the retention time is:

$$t_R = t_m(1 + FG) \tag{3}$$

where L is the column length and G the initial slope of the isotherm. The hold-up time increases with decreasing mobile phase velocity and the reaction proceeds for a longer period of time in the column. This allows the production of more hydrolyzate, i.e., A_2 becomes larger.

Effect of the Reaction Conditions

Parameters, such as substrate concentration, enzyme concentration, temperature, and pH exert the main influence on reaction rates. Figure 4 demonstrates the effect of temperature on the reaction chromatography of starch and

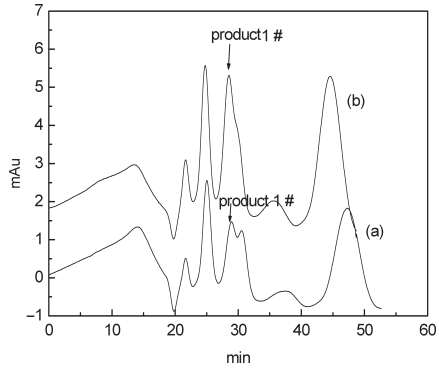

Figure 4. Influence of the reagent concentration and the process temperature. Experimental conditions: 10 × 2 cm i.d. column packed with 20 μm ODS silica; mobile phase, 0.24% (v/v) α-amylase solution; flow rate, 0.8 mL/min; column temperature, (a) for 50°C and (b) 60°C; sample, 1.0% (w/v) starch solution; injection amount, 20 μL.

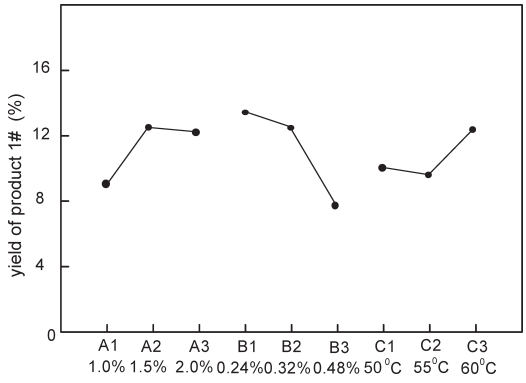

Figure 5. The efficiency of hydrolysis of starch in HPLC under different reactant concentrations (A), enzyme concentrations (B) and temperature (C).

α-amylase. The reaction rate at a certain temperature can be calculated using Eqn. (2) above, knowing A_2. For the results shown in Figure 1, the ratio of the reaction rate constants, k_r, for the enzyme and substrate concentrations were measured at 60°C and 50°C, respectively, approximated to 2.5. The effect of PH was not considered since the neutrality of water approaches the optimum pH for α-amylase activity.

An orthogonal test design was selected to evaluate the affects of: (A) starch concentration, (B) α-amylase, and (C) temperature on the enzymic hydrolysis within the HPLC column.[12] The results are shown in Figure 5. Limited by the difficulties in chromatographically resolving and determining the product #1, the three factors were varied through a restricted range. It was

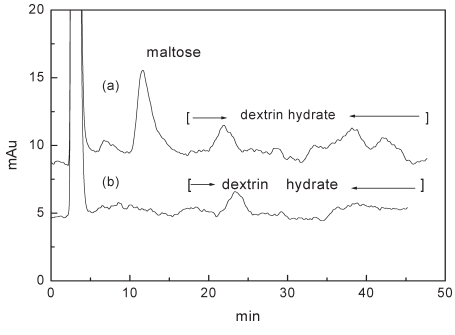

Figure 6. (a) Chromatogram of a standard maltose solution (5 mg/mL) and a dextrin hydrate solution (5 mg/mL). (b) Chromatogram of the cut fraction. Analysis conditions: Agilent 5 μm amino 150 × 4.6 mm packed analysis column; mobile phase, acetonitrile-water (80:20); flow rate, 1.0 mL/min; RI detector; column temperature, 25°C; sample, cut-off product #1; inject amount, 15 μL of (a) and 20 μL of (b).

determined that product yield has a limit, such that higher substrate and enzyme concentrations restrict the reaction. A higher concentration of starch solution will have a higher viscosity, which limits mass transfer within the column. Too high a concentration of α-amylase may result in a saturation effect, leading to a decrease in adsorption and desorption and affects column resolution. However, in accordance with the dynamic of mass transfer a higher column temperature provided increased reaction rates.

Analysis of the Product #1 of Reaction Chromatography of Starch

The enzyme α-amylase (α-1,4-D-glucan glucanohydrolase) hydrolyses the α-1,4 glucosidic bonds in starch, producing a range of maltooligosaccharides.[13] This reaction is complex and it is very difficult to analyze the maltooligosaccharides that are formed by the reaction.[14–16] To analysis the product #1, we collected the 2 mL fraction containing this product that elutes around 28.6 min, as shown in Figure 2, then boiling for 15 min to stop the reaction with α-amylase. The resultant denatured mixture was analyzed by high performance anion exchange liquid chromatography. The results are shown in Figure 6, where they are compared with the chromatograms of standard maltose and dextrin hydrate, which the products of starch hydrolysis should be.[13–15]

CONCLUSION

The separation conditions selected for chromatography play an important role in reaction chromatography, as was demonstrated in the experimental results reported.

When starch hydrolysis catalyzed by α-amylase is carried out by means of reaction chromatography, the process is more adjustable by using, for example, the flow rate or the particle rather than the concentrations or the temperature.

The experimental results indicate that carrying out starch hydrolysis in reaction chromatography could be an attractive alternative to conventional methods of preparation of dextrin hydrate, due to its flexibility.

ABBREVIATIONS

k'	capacity factor
N	number of theoretical plates of the column
C	concentration in the mobile phase, $\text{mol} \cdot \text{L}^{-1}$
f	concentration in the solid phase, $\text{mol} \cdot \text{L}^{-1}$
x	reduced distance along the column, m

t	time, min
k_r	reaction rate, s^{-1}
A	area of peak of the product
c_{10}	initial concentration of the reagent, $mol \cdot L^{-1}$
t_p	time of injection, min
L	column length, m
u	mobile phase linear velocity, cm/s
t_m	dead time, min

Greeks Symbol

ε	the total column porosity

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