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Journal of Chromatography B, 711 (1998) 309–312

JOURNAL OF CHROMATOGRAPHY B

Short communication

Separation and purification of glucoamylase in aqueous two-phase systems by a two-step extraction

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Abstract

Extraction in two steps of glucoamylase was studied in poly(ethylene glycol) (PEG) and potassium phosphate systems at pH values of 6, 7 and 9. Ten different conditions using PEG 300, 600, 1500, 4000 and 6000 were studied. The bottom phase of the first extraction step, with the enzyme, was reused in an appropriate concentration of PEG to form the second extraction step. The optimal partitioning conditions for glucoamylase separation were obtained in PEG 4000 (first step), PEG 1500 (second step) at pH 7 and resulted in a three-fold increase in glucoamylase purification.

Keywords: Aqueous two-phase systems; Glucoamylase; Enzymes

1. Introduction

Glucoamylase (E.C. 3.2.1.3) is an enzyme used in food and fermentation industries in the process of starch saccharification. There are several microorganisms that can produce glucoamylase, including bacteria, yeast and fungi, but *Aspergillus* and *Rhizopus* are the most used for commercial production [1].

Glucoamylase is produced by submerged fermentation and purified by traditional downstream processing. In this work, the separation of glucoamylase in an aqueous two-phase system of poly(ethylene glycol) (PEG) and potassium phosphate in two steps was studied, after an unsatisfactory attempt to purify the enzyme using a one-step extraction. An aqueous two-phase system (ATPS) is very attractive because it is easy to manipulate, can remove undesirable products in crude supernatants, is reliable in scaling-up and can substitute for conventional techniques [2,3].

An extraction involving two or more steps is applied when pigments and most of the contaminants need to be removed. Normally, the first step is used to remove pigments, cell debris and major contaminants, and the other steps are used to fractionate proteins in order to obtain maximum purification of the target protein [4].

2. Experimental

2.1. Chemicals

PEG 300 was supplied by Synth (São Paulo, Brazil). Potassium phosphate, PEG 600, 1500, 4000

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0378-4347/98/$19.00
PII: S0378-4347(98)00039-5

and 6000 (all of analytical-reagent grade) were obtained from Merck. Glucoamylase was produced in our laboratory from a submerged cultivation of *Aspergillus awamori* NRRL 3112.

2.2. Preparation of aqueous two-phase systems

Phase systems were prepared in 15 ml graduated centrifuge tubes by weighing the PEG (50%, w/w, for PEG 1500, 4000 and 6000, and 100%, w/w, for PEG 300 and 600) and a stock solution of phosphate (40%, w/w). The phosphate stock solution consisted of a mixture of appropriate amounts of KH_2PO_4 and K_2HPO_4 in order to obtain the desired pH. A 3-g mass of clarified broth that contained glucoamylase was added to the system, which was made up to 15 g by the addition of water. After phase separation, the appropriate mass of the bottom phase of the first step was added to a new system containing PEG and the system was made up to 5 g by the addition of water (second step). The total mass of the second step was 5 g, since the quantity of the first step's bottom phase was not sufficient. The quantity of the bottom phase from the first step added to the second step was calculated by the phase composition obtained from the corresponding diagram phase.

In both steps, the systems were vortex-mixed for 45 s and centrifuged at 1000 *g* for 5 min to speed up phase separation. Samples of top and bottom phases were assayed for total protein concentration, glucoamylase activity and by high-performance liquid chromatography (HPLC). Experiments were performed at 25°C.

2.3. Assays

Glucoamylase activity was determined by the amount of glucose produced by the action of enzyme. A 1-ml volume of sample (previously diluted) was added to 25 ml of starch solution (4%) at 60°C and pH 4.2 over a period of 60 min. The amount of glucose produced after 60 min was measured by the enzymatic glucose-oxidase method kit (Merck).

One unit of glucoamylase activity was the enzyme quantity that produced 1 g of glucose in 60 min at 60°C and pH 4.2.

Total protein concentration was measured by the Bradford assay [5]. Blank systems without protein were used as the reference.

HPLC was conducted using a GPC column (Protein Pak 125; Waters) of diameter 7.8 mm and length 300 mm. The composition of the mobile phase was 0.08 *M* phosphate buffer pH 7.0, 0.3 *M* NaCl and 20% (v/v) ethanol. The flow-rate was 0.5 ml/min. Previously, preparative gel permeation chromatography of clarified broth was done to obtain a pure enough sample of glucoamylase. This sample was used to identify the peak of glucoamylase in the HPLC analysis.

3. Results and discussion

Separation of glucoamylase by a two-step extraction was studied using combinations of PEG 300, 600, 1500, 4000 and 6000, according to Table 1. In all cases, glucoamylase had affinity towards the bottom phase in the first step, and impurities containing a yellow pigment present in the clarified broth were removed in the top phase.

Although the partition coefficient (*K*) of glucoamylase in systems with PEG 300 in the second step (systems 3 and 6) was high, the purification was poor, because most of the contaminants that were not removed in the first step also had affinity in the top phase. Similar results were observed in the PEG 600 system (system 4).

In all systems containing PEG 1500, 4000 and 6000, the values of *K* for glucoamylase and total proteins were low (between 10^{-2} and 10^{-4} in most cases for glucoamylase, and almost zero for total proteins), as shown in Table 1. These results showed that the separation of glucoamylase was poor since the contaminants and the enzyme had affinity towards the bottom phase. However, the HPLC profile of the bottom and top phases in both steps showed that glucoamylase was purified in the second step. A study of Lowry and Bradford methods showed that the Bradford method was not able to detect the majority of proteins present in the clarified broth used in the experiments [6,7] and, therefore, it was not possible to compare the partition coefficients of glucoamylase and total protein. Thus, HPLC was used to evaluate the partitioning of contaminants.

The retention time of glucoamylase was approxi-

Table 1
Experimental results

Run	Step	System composition	K_{GLU}	K_{Prot}	%GLU[a] Recovery	%[b] area
1	1	20% PEG 6000; 12% phosphate, pH 6.0	$4\cdot10^{-2}$	0	87.7	54.1
	2	15% PEG 1500; 15% phosphate, pH 6.0	$5\cdot10^{-4}$	0	~100	57.8
2	1	15% PEG 4000; 10% phosphate, pH 6.0	$3\cdot10^{-4}$	0	94.2	54.7
	2	15% PEG 1500; 12% phosphate, pH 6.0	$6\cdot10^{-4}$	0	~100	73.0
3	1	20% PEG 6000; 12% phosphate, pH 7.0	$3\cdot10^{-2}$	0	77.6	45.2
	2	25% PEG 300; 16% phosphate, pH 7.0	1399	∞	~100	50.3
4	1	20% PEG 6000; 12% phosphate, pH 7.0	$4\cdot10^{-2}$	0	~100	55.5
	2	12% PEG 600; 17.7% phosphate, pH 7.0	0.17	0	90.0	63.3
5	1	15% PEG 6000; 10% phosphate, pH 7.0	0	0	96.3	46.3
	2	15% PEG 1500; 10% phosphate, pH 7.0	$2\cdot10^{-3}$	0	~100	77.9
6	1	20% PEG 4000; 18% phosphate, pH 7.0	$9\cdot10^{-2}$	0	87.0	42.4
	2	23% PEG 300; 18% phosphate, pH 7.0	8.2	∞	57.8	49.9
7	1	15% PEG 4000; 15% phosphate, pH 7.0	$5\cdot10^{-2}$	0	88.9	44.8
	2	15% PEG 1500; 11% phosphate, pH 7.0	$2\cdot10^{-3}$	0	~100	65.9
8	1	14% PEG 4000; 10% phosphate, pH 7.0	$3\cdot10^{-4}$	0	~100	52.1
	2	15% PEG 1500; 12% phosphate, pH 7.0	$9\cdot10^{-4}$	0	84.4	73.1
9	1	14% PEG 4000; 10% phosphate, pH 7.0	$2\cdot10^{-4}$	0	~100	50.7
	2	20% PEG 1500; 8.5% phosphate, pH 7.0	$2\cdot10^{-3}$	0	96.1	81.2
10	1	15% PEG 6000; 10% phosphate, pH 9.0	$2\cdot10^{-3}$	0	~100	46.7
	2	13% PEG 1500; 9.6% phosphate, pH 9.0	$1\cdot10^{-2}$	0	~100	69.0

[a] Glucoamylase recovery: Relationship between enzymatic activity (U) in the phase where the highest glucoamylase concentration was obtained and the initial enzymatic activity (U).
[b] Percentage of the total area, determined by HPLC, representing glucoamylase.
GLU, glucoamylase

mately 13.65 to 13.85 min. Fig. 1 shows examples of the HPLC profiles obtained in the experiments. It is possible to see the differences between the clarified broth (before extraction) and the bottom phase after the second extraction. Fig. 1b shows that 73% of the total area of the HPLC profile represents the glucoamylase, which initially represented 28% of the total area. This percentage of total area was used to compare the partitioning of contaminants in the examined systems.

The percentage of total area shown in Table 1 was the value calculated by the integrator in the phase that contained more glucoamylase (bottom phase for systems with PEG 1500, 4000 and 6000, and top phase for systems with PEG 300 and 600).

The greatest purification yield of glucoamylase was obtained in system 9, which is very close to the critical point. It is not recommended to work with systems close to the critical point because of the instability. Thus, the best conditions for purifying glucoamylase were obtained in system 5, PEG 6000 (first step), PEG 1500 (second step) at pH 7, and this system gave results similar of those obtained using system 9.

4. Conclusion

Partitioning of glucoamylase in two consecutive steps of an ATPS gave good results in systems containing PEG 4000 and PEG 1500. The first step was used to separate the yellow pigment present in the clarified broth and the second step removed most of the remaining contaminants.

In the case of glucoamylase in a clarified broth, it is better to use high-molecular-mass PEGs (PEG 4000 or 6000) in the first step, to remove only pigments and substances with very low molecular masses (lower than 10 000), and PEG with a medium molecular mass (PEG 1000 or 1500), to remove the other contaminants, keeping the glucoamylase in the bottom phase.

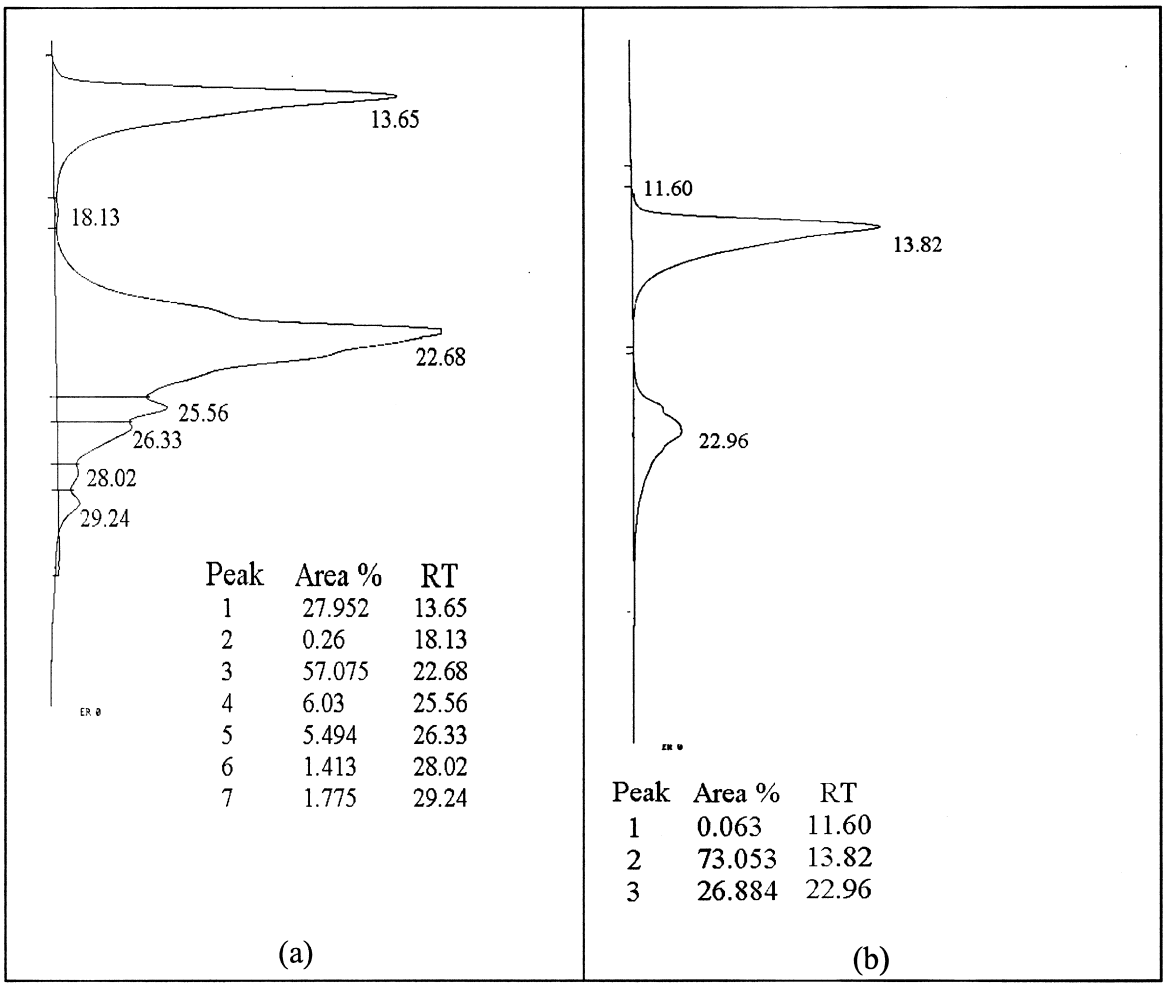

Fig. 1. Chromatographic profiles from the clarified broth (a) and bottom phase of the second step at pH 6, system 2 (b).

Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo.

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