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Journal of Chromatography B, 743 (2000) 241–246

JOURNAL OF CHROMATOGRAPHY B

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# Bioaffinity extraction of glucoamylase in aqueous two-phase systems using starch as free bioligand

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**Abstract**

Aqueous two-phase systems with bioligands bound to the polymer, usually polyethylene glycol (PEG), have high selectivity. However, such kind of systems can be costly since some specific synthetic ligands are expensive, making them non-viable for bioaffinity extraction of low-cost proteins. The use of free bioligands is an alternative for decreasing the high cost of these systems. This work describes the use of starch as a free bioligand on the partition of glucoamylase in PEG 300–phosphate system. The results were separated into two parts. In the first part, analysis of the starch distribution between the phases showed one-sided distribution to the bottom phase. In the second part, filtered broth from submerged cultivation of *Aspergillus awamori*, containing glucoamylase and contaminants, was submitted to the extraction in the system with starch. In the system without starch, glucoamylase and contaminants are partitioned in the upper phase. Upon starch addition, the partition coefficient of glucoamylase was decreased nine-fold without altering the partition of contaminants. These results indicate the possibility of separation of enzymes with high-molecular-mass and hydrophilic substrates, like glucoamylase, cellulase and pullulanase, from their contaminants in a one-step extraction. Since systems made of low-molecular-mass PEG partitioned almost all of the proteins to the upper phase, the separation can be achieved by extraction of the target enzyme in the bottom phase, as in the case of the presented study. 

*Keywords:* Aqueous two-phase systems; Partitioning; Enzymes; Glucoamylase; Bioligands, free

## 1. Introduction

The coefficient of partition of proteins in aqueous two-phase systems can be modified by addition of bioligands to the phase forming system. Free Cibacron Blue (a triazine dye used as a non-specific bioligand) in the 11% (w/w) polyethylene glycol (PEG) 2000–16.7% (w/w) Reppal PES 100 system, improved the coefficient of partition of lactate dehydrogenase from 0.45 to 1.55 [1]. Higher resolutions are possible by coupling a specific or non-specific bioligand to a one-phase forming polymer [2–5], usually PEG. Systems with coupled non-specific bioligands have been used for purification of a number of enzymes [4,6]. However, synthetic ligands for coupling specific bioligands can be costly, making these systems non-viable for low-cost proteins.

Hydrolytic enzymes, like glucoamylase, cellulase and pullulanase, act on substrates of high molecular mass and hydrophilic characteristics imposed by the hydroxyl radicals and reducer terminals of the sugar polymer. Therefore, such kinds of substrates are likely to have a low partition coefficient in PEG–salt systems. Moreover, if a low-molecular-mass PEG was employed, low- and high-molecular-mass contaminants could be directed to the upper phase while

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0378-4347/00/$ – see front matter 
PII: S0378-4347(00)00031-1

the target enzyme would be extracted in the bottom phase by bioaffinity to its substrate.

In this work, the separation of glucoamylase from clarified broth of *Aspergillus awamori* culture, by bioaffinity extraction using soluble starch as free bioligand, was studied.

## 2. Experimental

### 2.1. Chemicals

PEG 300 was supplied by Synth (São Paulo, Brazil). Soluble starch, mono- and dibasic potassium phosphates were obtained from Merck (Darmstadt, Germany). Glucoamylase was produced from a submerged cultivation of *Aspergillus awamori* NRRL 3112, according Zaldivar-Aguero et al. [7].

### 2.2. Experiments

Three sets of experiments were done in the PEG 300–phosphate system. In the first set, partitioning of starch (0.04 to 0.1%, w/w) was determined. In the second set, the influence of starch concentration on glucoamylase partitioning was determined. The influence of pH (6 and 7) on glucoamylase partitioning in the system added with starch was studied in the third set.

All the extractions were performed under a controlled temperature of 20°C through the use of a thermostatic water bath.

### 2.3. Preparation of aqueous two-phase systems

Phase systems were prepared in 15-ml graduated centrifuge tubes by weighing PEG 300, stock solution of starch (3%, w/v, in 0.006 *M* acetic buffer, pH 4.2) and phosphate buffer (40%, w/w, at pH 7 and 30%, w/w, at pH 6). Clarified broth containing glucoamylase was added to the second (4.0 g) and third (2.5 g) set of experiments. The systems were made up to 15 g by the addition of water, vortex-mixed for 45 s and centrifuged at 1000 *g* for 5 min at 20°C.

### 2.4. Assays

Glucoamylase activity was determined by the amount of glucose produced by the action of the enzyme. A 1-ml volume of sample previously diluted was added to 25 ml of starch solution (4%, w/v) at 60°C and pH 4.2 over a period of 60 min. The amount of glucose produced was measured by an enzymatic glucose-oxidase method kit supplied by Merck (Darmstadt, Germany). One unit of glucoamylase activity was the amount of enzyme that produced 1 g of glucose.

Proteins were analyzed by high-performance liquid chromatography (HPLC) using a gel permeation chromatography (GPC) column Protein Pak 300 SW supplied by Waters (MA, USA). The composition of the mobile phase (0.5 ml/min) was 0.08 *M* phosphate buffer, pH 7.0, 0.3 *M* NaCl and 20% (v/v) ethanol. A previous characterization of the clarified broth (retention time and molecular mass of glucoamylase and contaminants) was done in order to evaluate enzyme purity based on its relative area in the chromatographic analyses [9].

Starch concentration in the upper and bottom phases was determined through HPLC analysis conducted with an ion-exchange column Shodex SH 1011 supplied by Showa Denko (Tokyo, Japan). The composition of the mobile phase (0.8 ml/min) was 0.005 *M* $H_2SO_4$ and the column temperature was 50°C.

## 3. Results and discussion

Some care was necessary in the preparation of the systems to which starch was added. The large concentration of PEG in the system induces the formation of a solid–liquid phase system made of PEG and starch [1]. This precipitation of the starch leads to formation of a three-phase system. Hence, some precautions were taken: the composition of the experiments was chosen close to the critical point in order to employ the lowest concentrations of PEG and salt; after the addition of starch into the system, it was immediately vortex-mixed in order to minimize the contact time between the PEG and the starch solutions. Notwithstanding of these precau-

tions, some precipitation occurred because it was not possible to completely avoid the contact between the concentrated solutions of PEG and starch.

Through a preliminary set of experiments, it was verified that the coefficient of starch partition ($K_{st}$, relationship between concentrations) was strongly lower than 1, in systems with PEG 4000, 1500 and 300. The extraction of starch into the bottom phase confirms the hypothesis of the substrate partition, described in the Introduction. The PEG 300–phosphate system was chosen for the experiments since it extracts contaminants and glucoamylase to the upper phase [8]. The expected result is the maintenance of contaminants extraction in the upper phase while the enzyme will have its partition changed to the salt phase by action of starch.

### *3.1. Starch partition in the PEG 300–phosphate system*

The partition of 0.1, 0.07 and 0.04% (w/w) of starch was determined in the 15.50% (w/w) PEG 300–18.75% (w/w) phosphate, pH 7 system.

Fig. 1 shows that starch at 0.07 and 0.04% (w/w) presented a one-sided partitioning for the bottom phase, an interesting feature in order to explore the purification of the enzyme. Starch at 0.1% (w/w) presented a small partition (3.8% of starch) to the upper phase. The quantification of starch through HPLC, showed a total concentration in excess of about 30% in all three concentrations. Possibly, interactions of starch with phase compounds altered the refraction index of chromatographic analyses. Therefore, the quantification of precipitated starch by mass balance was impossible.

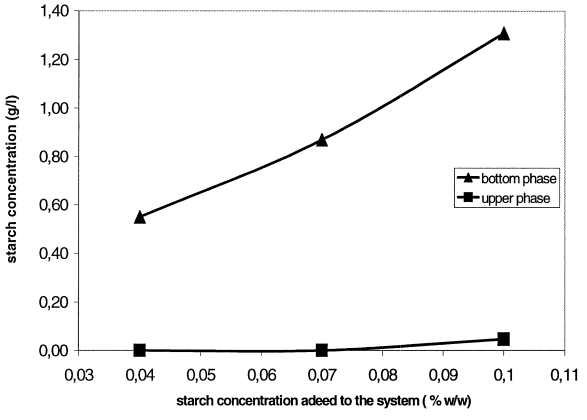


Fig. 1. Partition of starch in the 15.50% (w/w) PEG 300–18.75% (w/w) phosphate, pH 7 system.

### *3.2. Partition of glucoamylase in the PEG 300–phosphate–starch at pH 7 system*

The addition of starch into the PEG 300–phosphate system caused a loss of about 12% in total glucoamylase activity. Possibly, this loss was affected by an enzyme partition into the solid-phase. As this enzyme loss was almost constant, partition coefficients ($K_{GLU}$, relationship between enzyme activities in the liquid phases) were determined.

Fig. 2 shows that the addition of 0.1% (w/w) of starch to the same system described above (Section 3.1) leads to a nine-fold decrease in the $K_{GLU}$ value relative to a system without the bioligand. The increase of the glucoamylase activity in the bottom phase (3.8-fold) and the simultaneous decrease in the upper phase (2.5-fold), are depicted in the same figure. The maximum effective total starch concen-

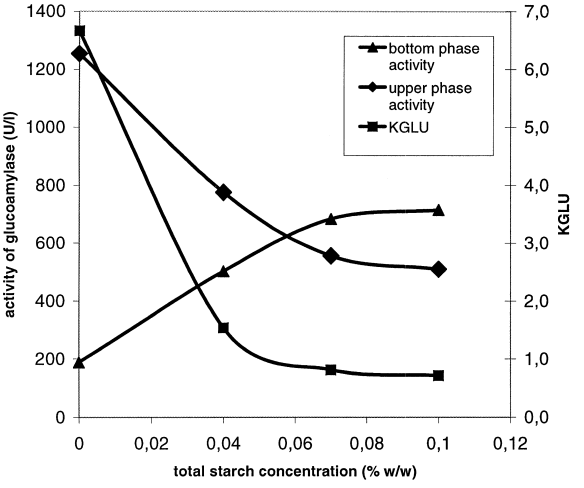


Fig. 2. Effect of starch addition on the partition of glucoamylase in the 15.50% (w/w) PEG 300–18.75% (w/w) phosphate, pH 7 system.

tration in this system was close to 0.07% (w/w). Starch concentrations higher than 0.07% did not further reduce $K_{GLU}$.

Another task of this extraction, was the maintenance of the contaminants partition into the upper phase. Fig. 3 shows chromatographic analyses of clarified broth and upper phase of an extraction with and without 0.1% (w/w) starch. Through this figure, it was possible to verify whether the partition of contaminants change upon 0.1% (w/w) of starch addition on the basis of the areas of each contaminants. These areas in the upper phase were almost not changed upon addition of starch into the PEG–phosphate system. The small changes (between 2% and 5%) can be attributed to imprecision in systems preparation. Therefore, the partition of contaminants was not changed.

The enzyme partition was severely changed through the addition of 0.1% (w/w) of the bioligand. Although, the maximum activity recovery in the bottom phase (53%) and the glucoamylase purity, measured as the relative area of the chromatographic analyses (23.1%), were still low and remains to be improved.

The product of starch hydrolysis (glucose) by glucoamylase was not found in the phases of system. Probably, this was influenced by the short time, about 6 min, and the low temperature of extraction process, 20°C, relative to the optimum temperature, 60°C, for glucoamylase action [10]. The minimal limit of glucose detection by the glucose-oxidase method is the quantity produced by hydrolysis of about 10% of the starch present in the bottom phase. Therefore, if some hydrolysis occurred, starch concentration was reduced by about less than 10% of the initial value.

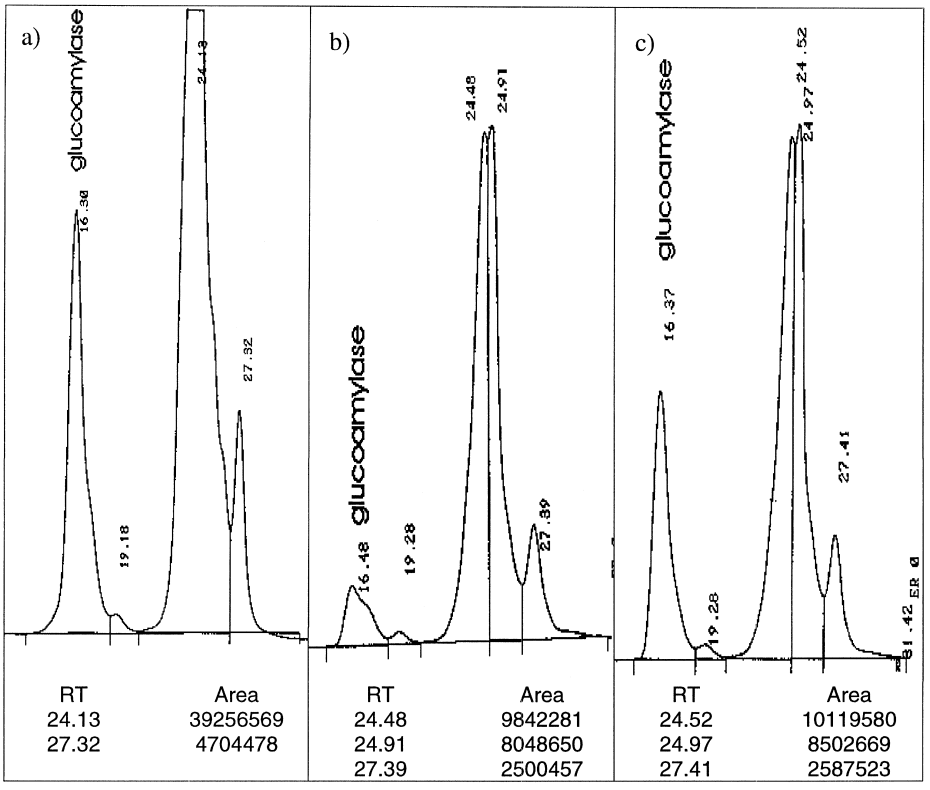


Fig. 3. Chromatograms, retention times (RT) and areas of contaminants in the clarified broth (a), upper phase of an extraction system with 0.1% of starch (b) and without starch (c).

Table 1
Influence of pH on the bioaffinity extraction[a]

| pH | $A_b$ (U/l) | $A_u$ (U/l) | $K_{GLU}$ |
|---|---|---|---|
| 6 | 477 | 242 | 0.51 |
| 7 | 388 | 277 | 0.71 |

[a] $A_b$: Bottom phase activity; $A_u$: upper phase activity.

### 3.3. Influence of pH in the glucoamylase partition in the PEG 300–phosphate–starch system

In this set of experiments, the initial composition was changed to 17.00% (w/w) PEG 300–19.00% (w/w) phosphate–0.1% (w/w) starch, in order to be in the biphasic region for both values of pH and as close as possible to the critical point.

Table 1 shows that as the pH value decreases from 7 to 6, glucoamylase activity in the bottom phase increases, i.e., the partition coefficient decreases. The optimum pH value for glucoamylase action is 4.5 [10], thus, the decrease in the partition coefficient may have occurred due to the increase of enzyme–substrate affinity. The loss of glucoamylase activity added into the system, in both pH values, was about 25%.

Extractions in this system under pH values lower than 6 were not possible because systems made with potassium phosphate salts, did not present phase separation at pH values below 6.

### 3.4. Influence of salting-out effect in the glucoamylase partition in the PEG 300–phosphate–starch at pH 7 system

The influence of salting-out effect was determined by addition of 2, 4 and 6% (w/w) of NaCl in the same system above described (Section 3.1).

The expected result through increasing the salting-out effect, was the decrease of the contaminant proteins concentration in the bottom phase. This would increase the glucoamylase purity in the bottom phase.

Fig. 4 shows that the increase of the salting-out effect decreases the glucoamylase activity in the bottom phase. This can be caused by decrease of the enzyme–starch affinity, by increase of starch precipitation or both.

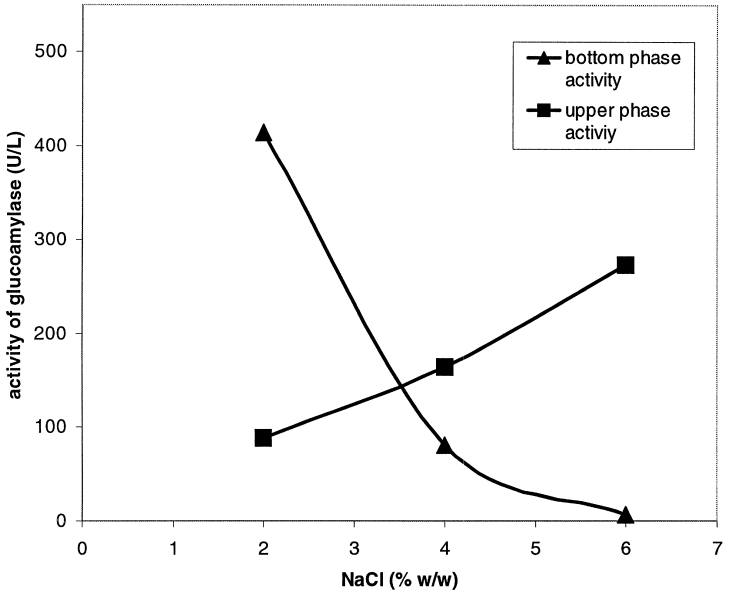


Fig. 4. Effect of increase in the salting-out effect in glucoamylase partition in the 15.50% (w/w) PEG 300–18.75% (w/w) phosphate, pH 7 system.

The addition of 2% of NaCl (w/w) to the system, decreased the glucoamylase activity recovery in the bottom phase (from 53% to 32%), and the glucoamylase purity, measured as the relative area of the chromatographic analyses (from 23.1% to 16.4%).

## 4. Conclusion

The addition of 0.1% (w/w) starch into the PEG 300–phosphate system reduced the partition coefficient of glucoamylase nine-fold (pH 7, 20°C). A higher reduction of $K_{GLU}$ was achieved at pH 6, possibly because this pH is closer to the optimum value for this enzyme than pH 7. The partitions of contaminants were not changed upon starch addition. They have affinity to the top phase for all the studied conditions. Therefore, the addition of starch in this system made possible the separation of enzyme from the contaminants.

The increase in the salting-out effect by addition of NaCl in the PEG 300–phosphate, pH 7 system, decreased the glucoamylase purity and activity recovery in the bottom phase.

These results indicate the possibility of separation of enzymes with high molecular mass and hydrophilic substrates, like glucoamylase, cellulase and pullulanase, from theirs contaminants, in one step extraction. Since systems made of low-molecular-mass PEG partitioned almost all of the proteins to the upper phase, the separation can be achieved by extraction of the target enzyme in the bottom phase, as in the case of the presented study.

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