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Journal of Chromatography A, 923 (2001) 275–280

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Short communication

Reversibly soluble macroaffinity ligand in aqueous two-phase separation of enzymes

Sunita Teotia, M.N. Gupta*

Chemistry Department, Indian Institute of Technology, Hauz Khas, New Delhi 110016, India

Received 9 January 2001; received in revised form 21 March 2001; accepted 14 May 2001

Abstract

Use of alginate as a free bioligand incorporated in an aqueous two-phase system of polyethylene glycol 6000–salt resulted in considerable purification of wheat germ α-amylase and sweet potato β-amylase from their crude extracts. The elution of the enzyme from the free bioligand was facilitated by exploiting the fact that alginate can be reversibly precipitated in the presence of Ca^{2+}. α-Amylase could be purified 42-fold with 92% activity recovery. β-Amylase on the other hand could be purified 43-fold with 90% recovery. Both purified enzymes showed a single band on sodium dodecylsulfate–polyacrylamide gel electrophoresis.

Keywords: Aqueous two-phase systems; Affinity adsorbents; Enzymes; Amylases; Alginates

1. Introduction

Partitioning in two-phase systems (consisting of polymer–polymer or salt–polymer) is an established technique for bioseparation of proteins/enzymes [1–3]. Its great advantage is that no preprocessing step is required for removal of particulate matter and one can directly carry out purification. Interfacing it with the affinity concept makes this technique rather selective as well. Such highly selective two-phase affinity extractions have been extensively described in the literature [4–8].

We have recently observed that alginate can act as a macroaffinity ligand for α-amylases [9,10] and β-amylases [11]. Alginate is a cheap, non-toxic polysaccharide of marine origin and consists of mannuronic acid and guluronic acid residues [12]. In this paper, we report that incorporation of alginate in a two-phase system of polyethylene glycol (PEG)–salt facilitates the purification of α-amylases and β-amylases.

2. Experimental

2.1. Chemicals

Sodium alginate (catalogue No. A-2158, composed predominantly of mannuronic acid residues) (abbreviated as HM alginate) and a PD-10 (Sephadex G-25) column were purchased from Sigma (St. Louis, MO, USA). PEG 6000 was supplied by Loba Chemie (Mumbai, India). Soluble starch was ob-

*Corresponding author. Tel.: +91-11-659-6599; fax: +91-11-658-1073.

E-mail address: mn_gupta@hotmail.com (M.N. Gupta).

0021-9673/01/$ – see front matter
PII: S0021-9673(01)00968-2

tained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Preparation of alginate solution

Alginate (2% w/v) solution was prepared in sodium acetate buffer (50 m*M*, pH 4.8 and 5.6) and stored at 4°C. This was diluted as per requirement prior to use.

2.3. Extraction of α-amylase from wheat germ powder

The α-amylase crude extract from wheat germ was prepared in 50 m*M* acetate buffer, pH 5.6 as described earlier [9].

2.4. Extraction of β-amylase from sweet potato

Crude extract of β-amylase from sweet potato was prepared in 50 m*M* acetate buffer, pH 4.8 as described by Bernfeld [13].

2.5. Estimation of α- and β-amylase activities

The activities were measured using starch as the substrate. One enzyme unit (U) is defined as the amount of enzyme which liberates 1 μmol of reducing sugar (calculated as maltose) per min at 25°C and pH 5.6/4.8 [13].

2.6. Protein estimation

Protein was estimated by the dye-binding method using bovine serum albumin as a standard protein [14].

2.7. Preparation of aqueous two-phase systems

Phase systems were prepared in graduated centrifuge tubes by mixing 22% (w/v) PEG 6000, 10% (w/v) dipotassium hydrogen orthophosphate, 10 and 12% (w/v) sodium chloride for α- and β-amylases, respectively. The two distinct phases were formed within 5 min. To this aqueous two-phase system 0.5% (w/v) and 0.6% (w/v) alginate was incorpo-

Table 1
Purification of α-amylase from wheat germ using aqueous PEG–salt and PEG/alginate–salt two-phase systems[a]

Steps	Activity (U)	Protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Fold purification
Crude extract	42.2	4.6	9.2	100.0	1.0
Bottom Phase (salt)					
No alginate	16.9	2.8	6.0	40.0	0.7
+alginate	0.8	3.1	0.3	1.9	0.0
Top Phase (PEG)					
No alginate	22.8	0.4	57.0	54.0	6.2
+alginate	40.1	0.4	100.2	95.0	10.9
Supernatant +wash of alginate	1.3	0.3	4.3	3.1	0.5
Eluate	38.8	0.1	388.0	91.9	42

[a] The separation of phases was done as described in the Experimental section. In the case of PEG/alginate–salt system, elution of the partitioned activity from the top phase was done by incubating the enzyme-bound alginate with 1.0 ml of acetate buffer (50 m*M*, pH 5.6) containing 1.0 *M* maltose at 4°C for 4 h. The activity was determined after removal of maltose using a PD-10 column from the supernatant. All the experiments were performed in duplicate and the difference in the readings in the duplicates was less than ±5%.

rated in the case of α- and β-amylases, respectively. Thereafter, crude preparations of α-amylase (1.0 ml containing 42.2 U) and β-amylase (1.25 ml containing 58.5 U) were added to the corresponding systems. Alginate distribution was restricted to the PEG phase with less than 5% (of the initially added amount) going to the bottom phase. Alginate concentration in the two phases was estimated by the phenol–sulphuric acid method [15]. The two phases were separated. The alginate in the top phase was precipitated in the presence of 70 m*M* Ca^{2+} and incubated for 20 min at 25°C. The precipitate was centrifuged at 8000 *g* for 10 min at 25°C. The supernatant and subsequent washings with buffer (until no enzyme activity was detected in the washings) were collected. The amount of partitioned α- and β-amylases were calculated by the difference of initial activities and activities recovered in supernatant and washings.

Thus, the purification of α- and β-amylases was carried out using alginate concentration of 0.5 and 0.6% (w/v), temperature 25°C and at pH 6.0 and 5.5, respectively.

2.8. Polyacrylamide gel electrophoresis (PAGE)

Sodium dodecylsulfate (SDS)–polyacrylamide gel (12%) was performed following the procedure of Hames [16] using Hoefer Mighty Small II and a SE 250 Mini-Vertical Gel Electrophoresis Unit (Amersham Pharmacia Biotech, USA).

3. Results and discussion

The α-amylase activity from wheat germ gets evenly distributed in the PEG and salt phases (Table 1). However, the specific activity of the enzyme in

Table 2
Purification of β-amylase from sweet potato using aqueous PEG–salt and PEG/alginate–salt two-phase systems[a]

Steps	Activity (U)	Protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Fold purification
Crude extract	58.5	4.7	12.4	100.0	1.0
Bottom Phase (salt)					
No alginate	27.8	1.5	18.5	47.5	1.5
+alginate	1.5	1.8	0.8	2.5	0.1
Interface					
No alginate	2.9	2.3	1.3	5.0	0.1
+alginate	0.5	2.0	0.3	0.9	0.0
Top Phase (PEG)					
No alginate	25.5	0.1	255.0	43.6	20.6
+alginate	56.2	0.3	187.3	96.0	15.0
Supernatant +wash of alginate	0.7	0.1	7.0	1.2	0.6
Eluate	52.7	0.1	527.0	90.0	43

[a] The separation of phases was done as described in the Experimental section. In the case of PEG/alginate–salt system, elution of the partitioned activity from the top phase was done by incubating the enzyme-bound alginate with 1.0 ml of acetate buffer (50 m*M*, pH 4.8) containing 1.0 *M* maltose at 4°C for 4 h. The activity was determined after removal of maltose using a PD-10 column from the supernatant. All the experiments were performed in duplicate and the difference in the readings in the duplicates was less than ±5%.

the PEG phase is higher since bulk of the protein partitions into the salt phase. The latter phenomenon is in agreement with the general behavior of proteins in such two-phase systems [1,17]. Incorporation of alginate in PEG phase resulted in greater amount of α-amylase appearing in this phase. Alginate concentration was varied between 0.1 and 0.9%. The maximum partitioning (95% of total activity started) of α-amylase was observed with 0.5% (w/v) alginate (Table 1). Elution of this alginate bound activity resulted in 92% yield with 42-fold purification. Thus, the presence of alginate as a macroaffinity ligand dramatically enhanced the partition of the α-amylase activity into the PEG phase.

The robustness of two-phase extractions permits one to directly deal with unclarified crude extracts. When crude and unclarified (without centrifugation) α-amylase extract was used, there was only marginal decrease in activity recovery (7% decrease) and fold purification (3% decrease).

As alginate is also known to have affinity for β-amylase [11], similar experiments were carried out with sweet potato β-amylase. Table 2 shows that a larger fraction of β-amylase activity partitions in the salt phase. Here also, incorporation of alginate at varying concentrations was tried and maximum partitioning of 94% of β-amylase was found in PEG phase when 0.6% (w/v) alginate was present in (Table 2) PEG phase. After elution from alginate, an activity recovery of 90% with 43-fold purification was obtained. Again, when working with crude unclarified extracts, activity recovery of 82% and fold purification of 35 were obtained.

The best load of α-amylase activity which could be processed by alginate containing two-phase system was found to be 42 U. While the recovery in all the cases was in the range of 92–97% of the partitioned activity, a load beyond 42 U decreased the initial binding of the α-amylase activity to alginate. This behavior is rather well known and is explained by the fact that crowding on the matrix decreases the binding of macromolecules to polymer surfaces [18,19]. Similar behavior was observed in the case of sweet potato β-amylase. Here, 58.5 U of activity could be processed with the chosen parameters of the system.

The dependence of the partitioned activity on the pH of the aqueous phase in the case of α- and β-amylase was determined. pH 6.0 was best for α-amylase and pH 5.5 for β-amylase was found to give best results (Below pH 5.5, the phase formation was not observed). Fig. 1 shows the change in fold purification with sodium chloride concentration in the aqueous phase, 10 and 12% NaCl concentrations were found to be optimum for α- and β-amylases respectively.

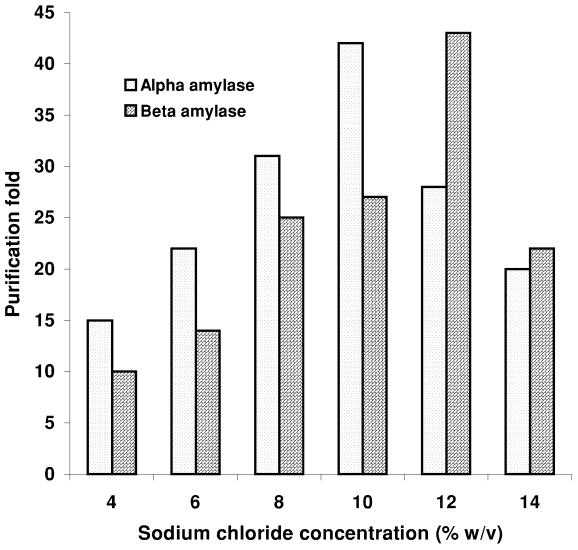

Fig. 1. Effect of increase in the sodium chloride concentration on the purification fold of α- and β-amylase in the aqueous two-phase system containing 22% (w/v) PEG, 10% (w/v) K_2HPO_4 at pH 6.0/5.5, respectively. The optimum concentration of sodium chloride in the systems were 10 and 12% for α- and β-amylases.

The SDS–PAGE patterns of crude and purified preparation of wheat germ α-amylase and sweet potato β-amylase are given in Fig. 2A and B. In both cases the purified preparations show a single band. The molecular weights for the purified enzymes reflected in Fig. 2 agree with the values reported earlier [10,20].

4. Conclusion

An added advantage of using this approach is that one does not have to separate PEG from the purified protein, as the protein binds to alginate. Alginate is a fairly inexpensive polysaccharide but, in principle, it could be recycled. The approach used in this work,

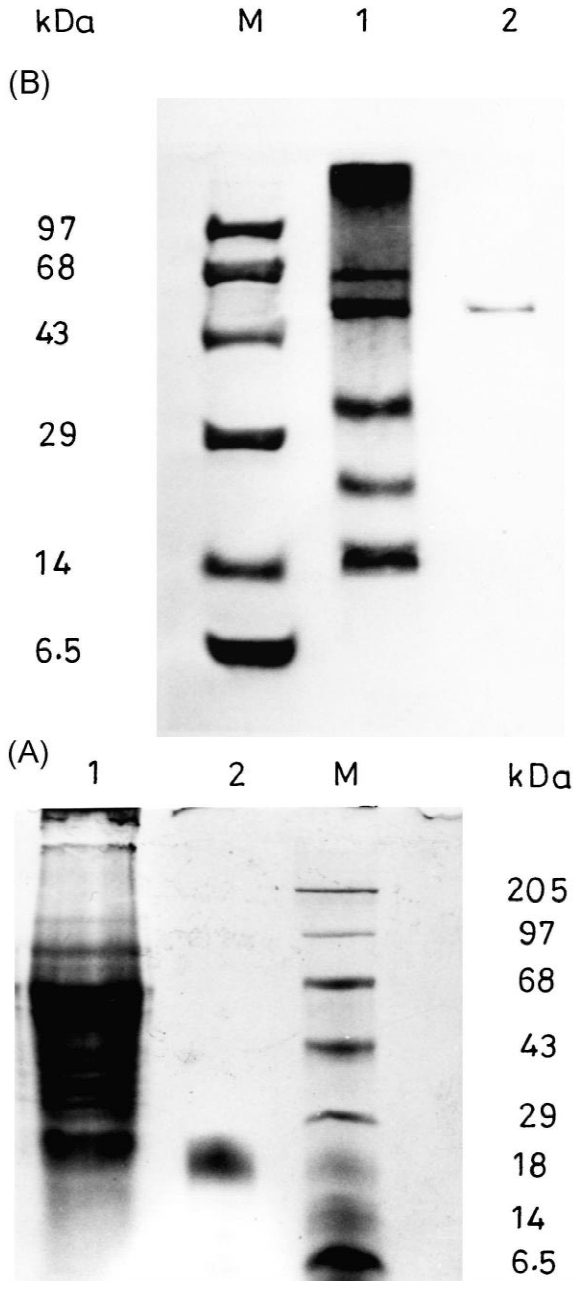

Fig. 2. (A) SDS–PAGE pattern of wheat germ α-amylase. Lanes: 1=crude wheat germ extract; 2=purified protein preparation; 3=marker proteins. (B) SDS–PAGE pattern of sweet potato β-amylase. Lanes: 1=marker proteins; 2=crude sweet potato extract; 3=purified protein preparation.

in fact, was based upon an earlier work by Kamihara et al. [21], who had incorporated IgG coupled to Eudragit S-100 (a polymer of methacrylic acid and methylmethacrylate) in PEG–Reppal PES 200 two-phase systems for affinity extraction of recombinant protein A. While the present work was in progress, we have come across a report by Gouveia and Kilikian in which they purified glucoamylase using starch as free bioligand in PEG–salt system [8]. Thus, the approach outlined here and the results obtained show that incorporation of a free macroaffinity ligand in two-phase systems can be a powerful technique for bioseparation of enzymes/proteins. Our work, in fact uses alginate as reversibly soluble–insoluble macroaffinity ligand which make the process more convenient for enzyme recovery.

Acknowledgements

This work was supported by Council for Scientific and Industrial Research (Technology Mission on Oilseeds, Pulses and Maize Project), India. The financial support of the organization to S.T. in the form of SRA (senior research associate) is also acknowledged.

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