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Carbohydrate Polymers 50 (2002) 63–68

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Partitioning of pectinolytic enzymes in polyethylene glycol/potassium phosphate aqueous two-phase systems

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Received 20 July 2001; revised 6 December 2001; accepted 6 December 2001

Abstract

Four different pectinolytic enzymes have been partitioned in polyethylene glycol (PEG)-potassium phosphate aqueous two-phase systems (20–15%). The influence of PEG molecular weight and the addition of sodium chloride were investigated. The best results concerning to the partition coefficients (K_p and K_e) and the yield recovery (R) were: PEG-400/phosphate with NaCl for *exo*-polygalacturonase ($K_e = 5.35$ and $R = 89.5\%$), PEG-600/phosphate for pectin lyase (PL) ($K_e = 43.18$ and $R = 98.5\%$) and pectinesterase (PE) ($K_e = 1.51$ and $R = 69.6\%$), and PEG-10,000/phosphate for *endo*-polygalacturonase ($K_e = 1.35$ and $R = 53.5\%$). The best purification factors were observed in the upper phase for the systems containing high molecular weight PEG without NaCl: PEG-6000 for *exo*-polygalacturonase (5.49 fold) and PEG-10,000 for *endo*-polygalacturonase (16.28 fold), PE (16.64 fold) and PL (14.27 fold).

Keywords: Pectinase; Commercial enzyme; Partitioning; Aqueous two-phase systems

1. Introduction

Pectinase is a commercial preparation of pectinolytic enzymes that degrades the pectin molecule. Its main components are: Pectinesterase (PE) that removes methoxyl groups from methylated pectin substances, pectin lyase (PL) which splits the glycosidic bond by β-elimination of hydrogen from the C-4 and C-5 position of the aglycone portion of the substrate and polygalacturonases (PG) that are classified into *exo*-PG and *endo*-PG. *Exo*-PG acts on the non-reducing end of the substrate chain, while *endo*-PG has a random action within the chain, both breaking the glycosidic bonds (Channe & Shewale, 1995; Ros, Nuñez, Saura, Salmeón, & Laencina, 1991; Whitaker, 1984).

Pectinolytic enzymes are important for the treatment of raw fibres in the textile industry, clarification of juices and wine, maceration of vegetables and fruits and extraction of olive oil (Baracat, Valehtin, Muchovej, & Silva, 1989; Kotzekidou, 1991; Manachini, Parani, & Fortina, 1988; Pilnik & Voragen, 1970).

The commercial pectinase preparations used in the industry often come from fungal source and normally contain a mixture of pectinolytic enzymes associated with hemicellulases and/or cellulases (Kotzekidou, 1991). But there are processes requiring only one type of pectinase, such as the use of PE for clarifying cider or *endo*-PG for preparing orange and citrus juices (Manachini et al., 1988).

For the success of the commercial production of enzymes and proteins, there is a need for efficient downstream processing techniques. The downstream processing of these biological materials requires purification techniques that are delicate enough to preserve the biological activity (Tanuja, Srinivas, Rao, & Gowthmann, 1997). The purification protocols involve several steps, which increase the cost of the process and reduce the yield. The conventional procedures include ammonium sulphate precipitation, chromatography, dialysis and filtration. Simpler and more efficient purification processes are needed.

Aqueous two-phase systems (ATPS) could be a good alternative to a first purification step since such systems allow removal of several contaminants by a simple and economic process. ATPS are formed by adding to water, either two structurally different hydrophilic polymers, such as dextran and polyethylene glycol (PEG) (Marcos, Fonseca, Ramalho, & Cabral, 1999), or maltodextrin and PEG (Silva & Meirelles, 2000a), or a polymer and salt, such as PEG and potassium phosphate or PEG and sodium sulphate (Silva & Meirelles, 2001; Silva, Coimbra, & Meirelles, 1997; Snyder, Cole, & Szlag, 1992). Phase equilibrium data for such systems are mainly found in the works of Albertsson (1986) and Zaslavsky (1995).

There are many reports in the literature concerning to the

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0144-8617/02/$ - see front matter
PII: S0144-8617(01)00376-9

Table 1
PEG characterisation

PEG	Average molecular weight	Polydispersity index
1450	1468	1.03
3350	2938	1.04
8000	8768	1.09
10,000	11,589	1.10

partition of different enzymes and proteins in ATPS (Alves, Chumpitaz, Silva, Franco, & Meirelles, 2000; Huddleston, Abelaira, Wang, & Lyddiatt, 1996; Marcos et al., 1999; Silva & Meirelles, 2000b; Silva & Meirelles, 2001, Vernau and Kula, 1990), but reports on the use of such systems for partitioning and purification of pectinolytic enzymes were not available.

In the present work, we report the partitioning of four pectinolytic enzymes from a commercial pectinase preparation (Pectinex-3XL®) in ATPS composed by PEG and potassium phosphate. The influence of PEG molecular weight and the addition of sodium chloride were investigated.

2. Materials and methods

PEGs with molecular weight of 400, 600, 1000, 1450, 3350, 8000 and 10,000 were purchased from Sigma. The polydispersity indexes and average molecular masses for PEGs 1450, 3350, 8000 and 10,000 were determined by gel permeation chromatography (GPC) using an Ultrahydrogel column Waters device. The following experimental conditions were employed for the GPC runs: Water as the mobile phase at a rate of 0.8 μl min^{-1}, injection temperature of 313.15 K, volume of the sample injected equal to 100 μl, and a refractive index detector. The characteristics of those PEGs are given in Table 1. For low molecular weight PEGs the average molecular mass is usually very close to that specified by the PEG denomination (for example: PEGs 200, 400, 600 and 1000), as indicated in the literature (Cruz, Chumpitaz, Alves, & Meirelles, 2000). For this reason the polymer characterisation was not performed for the low molecular weight PEGs.

Pectin (partially methoxilated polygalacturonic acid) from citrus fruits, containing 85% of galacturonic acid and 10% of methoxy groups, was purchased from Sigma. Potassium phosphate monobasic, potassium phosphate dibasic, sodium citrate and sodium chloride were purchased from Sinth (Diadema, SP, Brazil), calcium chloride, sulphuric acid and acetic acid was purchased from ECIBRA (Santo Amaro, SP, Brazil). The reagents used in this work had analytical grade. The commercial enzyme (Pectinex-3XL®) was kindly supplied by New Nordisk (Araucária, PR, Brazil). According to the manufacturer this commercial enzyme contained PE, PL, *endo*-PG, *exo*-PG, cellulase and hemicellulase.

2.1. Aqueous two-phase systems

The systems were prepared from stock solutions of PEG (50%, w/v), phosphate buffer, pH 7.0 as a mixture of potassium phosphate monobasic, potassium phosphate dibasic (50%, w/v) in a ratio of 1.087 (w/w), and sodium chloride (30%, w/v), stored at 4 °C. The quantity of the commercial enzyme preparation added to the systems was always 10% of the total system volume (12 ml), and was the last component added to the system. All systems were prepared in graduated centrifuge tubes. After 2 min of gently stirring, the systems were centrifuged (BR4i model) at 4 ± 0.1 °C and 2900 g for 10 min. The tubes were brought to equilibrium in a thermostatic bath (Marconi MA-184) at 4 °C and local atmospheric pressure (727 mmHg) for overnight (at least 12 h). After this treatment, the two phases become clear and transparent, and the interface was well defined. The phases were carefully withdrawn using a pipette for the upper phase and a syringe with a long needle for the bottom phase. The volumes were determined in graduated test tubes.

The partition coefficient is defined as the protein concentration or enzyme activity in the upper phase divided by the correspondent value in the bottom phase, as shown in Eqs. (1) and (2).

$$K_P = \frac{C_T}{C_B} \tag{1}$$

$$K_E = \frac{A_T}{A_B} \tag{2}$$

where C_T and C_B are the total protein concentration in mg/ml of the upper and bottom phases, respectively, and A_T and A_B is the enzyme activity in the upper and bottom phases, respectively.

In order to evaluate the purification process the enzyme specific activity (SA, expressed in U/mg protein), the purification factor (PF) and the enzyme yield recovered in the upper (R_T) and bottom phases (R_B) were also calculated, according to the given equations:

$$SA = \frac{A}{C} \tag{3}$$

$$PF = \frac{SA}{SA_i} \tag{4}$$

$$R_T = \frac{100}{1 + \dfrac{1}{R_v K_E}} \tag{5}$$

$$R_B = \frac{100}{1 + R_v K_E} \tag{6}$$

$$R_v = \frac{V_T}{V_B} \tag{7}$$

Table 2
Partition coefficients in systems PEG-phosphate at 4 °C and pH 7.0

PEG[a]	*Endo*-PG	*Exo*-PG	PL	PE	Protein
400	1.09 ± 0.02	1.86 ± 0.03	6.43 ± 0.60	1.33 ± 0.08	0.82 ± 0.04
600	1.04 ± 0.03	0.67 ± 0.04	43.18 ± 14.54	1.51 ± 0.17	0.54 ± 0.05
1000	1.13 ± 0.06	0.16 ± 0.02	1.98 ± 0.04	0.78 ± 0.09	0.10 ± 0.03
1450	0.81 ± 0.04	0.24 ± 0.04	1.76 ± 0.04	0.70 ± 0.05	0.11 ± 0.00
3350	0.78 ± 0.14	0.08 ± 0.00	0.86 ± 0.01	0.62 ± 0.02	0.06 ± 0.00
8000	0.84 ± 0.05	0.46 ± 0.02	0.51 ± 0.02	0.70 ± 0.05	0.07 ± 0.02
10,000	1.35 ± 0.20	0.18 ± 0.00	0.86 ± 0.08	1.14 ± 0.01	0.04 ± 0.00

[a] Molecular weight.

where R_v is the volume ratio, and V_T and V_B are the volumes of upper and bottom phases, respectively.

The enzyme specific activity (SA) can be evaluated for both phases, using Eq. (3) with the corresponding enzyme activity and the protein concentration in the selected phase. In Eq. (4), SA_i represents the SA for the initial sample (before extraction).

The partition experiments were carried out in triplicate and the average results are the values reported in the present work.

2.2. Enzyme assays

PL activity was determined spectrophotometrically (Beckmann DU-70) by monitoring the increase in the A_{235} as described by Albersheim and Killias (1962). The reaction mixture is composed by 1.25 ml of citrate-phosphate (0.15 M) buffer, pH 5.5, 1.0 ml of pectin solution (1% w/v) and 0.25 ml of enzyme sample. The mixture was incubated for 60 min at 4 °C and the reaction was stopped by incubation for 10 min in a water bath at 100 °C. One unit of PL is defined as the amount of enzyme, which produces an increase of one unit of A_{235} per minute. PE activity was assayed by quantification of methanol (Wood & Siddiqui, 1971). The reaction mixture consisted of 1.0 ml of pectin solution (1.0% w/v), 2.0 ml acetate (1.0 M) buffer, pH 7.5, adjusted with acetic acid, and made 0.002 M in $CaCl_2$ and 1.0 ml of enzyme solution. The reaction was incubated for 60 min at 25 °C and was stopped by addition of 4.0 ml of H_2SO_4 (1.0 M). One unit of PE is defined as the amount of enzyme that produced 1 μmol methanol per minute. *Exo*-PG activity was measured via the reducing groups released in the reaction mixture by the Somogy–Nelson procedure described by Baracat-Pereira, Vanetti, Araújo, and Silva (1993) and Kotzekidou (1991). The reaction mixture contained 1.0 ml acetate (0.1 M) buffer, pH 4.5 and 0.5 ml enzyme solution. The incubation was carried out at 37 °C for 60 min. The reaction was stopped by incubation for 10 min in a water bath at 100 °C. One unit of *exo*-PG is the amount of enzyme that released 1 μmol galacturonic acid per minute. *Endo*-PG activity was determined by the decrease in relative viscosity of the reaction mixture composed by 18.0 ml of pectin solution (2% w/v) in acetate (0.1 M) buffer, pH 4.5 and 2.0 ml of enzyme solution, using a viscometer (Brookfield LVDV-II +). The reaction was stopped by incubation for 10 min in a water bath at 100 °C. One unit of *endo*-PG is defined as the amount of enzyme that promotes a reduction of 1% in initial viscosity per minute. In all enzyme assays control tubes, containing the enzyme sample previously inactivated by incubation for 10 min in a water bath at 100 °C, were used. Protein was determined by the Bradford method (1976).

3. Results and discussion

3.1. Influence of PEG molecular weight

The values of the partition coefficients as a function of PEG molecular weight are given in Table 2. The studies were conducted at pH 7.0 and 4 °C. The standard deviation for the partition coefficients varied between 0.052 and 0.001 for protein, 0.170 and 0.009 for PE, 0.011 and 14.540 for PL, 0.040 and 0.001 for *exo*-PG and 0.200 and 0.020 for *endo*-PG. For the partition coefficient of PL measured in the system with PEG-600 the standard deviation was 14.54. In this case the enzyme activity in the salt phase was too small (0.16 U ml^{-1}) making difficult the evaluation of its value by the selected method. For this reason the standard deviation for the bottom phase activity was high.

The partition coefficients were significantly influenced by the PEG molecular weight. For protein it decreased as the PEG molecular weight increases, but it varied always within the range 0.82–0.04. A similar behaviour was observed by Lahore, Miranda, Fraile, Bonino, and Cascone (1995) in the partition of acid protease from *Mucor bacilliforms*. Most pectinolytic enzymes present higher affinity for the PEG-rich phase, especially when PEG of low molecular weight was utilised. The best values were 1.51 for PE, 43.18 for PL in PEG-600 and 1.86 for *exo*-PG in PEG-400. But the partition coefficients of *endo*-PG varied within the range 1.35–0.78, the highest value being observed for the system with PEG-10,000. Engel, Barak, Chipman, and Merchuk (2000) obtained higher partition coefficients for acetohydroxy acid synthase at lower PEG molecular weights. Tanuja et al. (1997) also reported a similar

Table 3
Yield recovery (%) in the upper phase in systems PEG-phosphate at 4 °C and pH 7.0

PEG	*Endo*-PG	*Exo*-PG	PL	PE	Protein
400	72.2	81.5	94.5	76.0	67.2
600	61.2	50.5	98.5	69.6	41.2
1000	59.0	16.9	71.6	49.8	9.1
1450	47.5	20.8	66.2	43.7	10.2
3350	44.6	8.0	47.2	39.2	4.7
8000	42.4	28.7	30.9	38.3	5.4
10,000	53.5	13.3	42.3	49.1	2.8

Table 4
PF in the upper phase in PEG-phosphate systems at 4 °C and pH 7.0

PEG	*Endo*-PG	*Exo*-PG	PL	PE
400	1.09	1.23	1.42	1.15
600	1.37	1.13	2.19	1.54
1000	5.10	1.55	6.49	4.48
1450	4.27	1.92	5.97	3.94
3350	8.27	1.46	8.59	7.13
8000	6.84	4.79	5.14	6.38
10,000	16.28	4.50	14.27	16.64

behaviour for amyloglucosidase. In the present work the same trend was obtained for most pectinases.

It should be considered that bio-molecule partitioning in ATPS is a complex function of a variety of factors, including the bio-molecule size, its surface properties, net charge, the system pH and temperature and the polymer molecular weight (Baskir, Hatton, & Suter, 1989). Usually the partition coefficient decreases as the PEG chain length increases, behaviour also observed in the present case for the proteins and most pectinolytic enzymes. As the bio-molecule size increases, its preference for partitioning into one phase also tends to increase. The partitioning behaviour is also influenced by the protein or enzyme net charge. Unfortunately there is not much information about such properties in the case of pectinolytic enzymes. Whitaker (1984) reports that PL has an isoeletric point of 9.0, so that its net charge in the systems used in the present work (pH 7.0) should be slightly positive. According to Whitaker (1984) the molecular weights of PL and PG are in the range 30–50 and 30–40 kDa, respectively. Considering that such molecular weight ranges are not significantly different, the bio-molecule sizes may eventually not explain the differences in the partitioning behaviour of those enzymes. A third aspect that should be taken into account is the relative hydrophobicity of the enzyme surface. For bio-molecules with similar size and net charge, a higher presence of hydrophobic regions at the molecule surface tends to enhance its preference for the polymer phase, an aspect that eventually can also explain the differences in the partitioning behaviour of the pectinolytic enzymes observed in the present work.

The results of yield recovery in the upper phase were shown in Table 3. In most cases the enzyme yield recovery in the upper phase were higher than 50% for systems containing PEG of low molecular weight. The yield recovery in the bottom phase increased for higher PEG molecular weight. This was specially pronounced in the case of *exo*-PG. The protein recovery in the upper phase decreased as the PEG molecular weight increases.

The PF of pectinolytic enzymes as a function of PEG molecular weight can be seen in Table 4. The PFs were higher in the upper phase, indicating that purification of pectinases should be performed in this phase. The best results were observed in PEG-8000 for *exo*-PG (4.79 fold) and in PEG-10,000 for the other enzymes (*endo*-PG-16.28 fold, PL-14.27 fold and PE-16.64 fold). It should be noted that such high values of the PFs in the upper phase were obtained for systems with PEG of higher molecular weight because in these systems the proteins have a very strong affinity for the bottom phase. In fact, the trend of the partition coefficients to decrease as the PEG molecular weight increases is much more pronounced in the case of proteins than for the enzymes. For the partition of penicillin acylase from *Escherichia coli* in PEG-sodium citrate systems, Marcos et al. (1999) obtained the best results using PEG-1000: 83% and 2.6 fold for yield recovery and PF, respectively. In the present work the PF for most pectinolytic enzymes is significantly higher than that obtained by Marcos et al. (1999), but the yield recovery is lower. Polizeli, Jorge, and Terenzi (1991) studied the purification of pectinolytic enzymes using precipitation with ethanol and three chromatographic steps. They obtained purifications factors for the overall process equal to 56.14 fold for *exo*-PG and 20.73 fold for PL. The corresponding yield recovery were 28.0% and 5.96%, respectively. In the present case a PF of 14.27 and a yield recovery of 42.3% were obtained for PL in only one liquid–liquid extraction step using the

Table 5
Partition coefficients in PEG-phosphate systems with 6% (w/v) of NaCl at 4 °C and pH 7.0

PEG	*Endo*-PG	*Exo*-PG	PL	PE	Protein
400	1.15 ± 0.11	5.35 ± 0.22	7.64 ± 0.01	1.34 ± 0.00	1.43 ± 0.02
600	1.01 ± 0.00	0.94 ± 0.08	9.55 ± 0.03	0.99 ± 0.04	0.67 ± 0.01
1000	0.90 ± 0.03	0.22 ± 0.05	2.66 ± 0.00	0.67 ± 0.00	0.24 ± 0.00
1450	0.65 ± 0.02	0.15 ± 0.02	1.40 ± 0.01	0.63 ± 0.01	0.10 ± 0.00
3350	0.80 ± 0.03	0.16 ± 0.05	1.33 ± 0.01	0.65 ± 0.01	0.07 ± 0.01
8000	0.48 ± 0.01	0.39 ± 0.04	1.15 ± 0.01	0.71 ± 0.07	0.17 ± 0.01
10,000	0.85 ± 0.05	0.74 ± 0.03	1.18 ± 0.04	0.85 ± 0.01	0.17 ± 0.02

Table 6
Yield recovery (%) in the upper phase in PEG-phosphate systems with 6% (w/v) of NaCl at 4 °C and pH 7.0

PEG	*Endo*-PG	*Exo*-PG	PL	PE	Protein
400	64.6	89.5	92.4	68.1	69.5
600	56.8	55.1	92.5	56.2	46.6
1000	49.1	18.8	74.0	42.0	20.2
1450	40.4	13.1	59.2	39.3	9.0
3350	43.5	13.6	56.3	38.4	6.5
8000	28.0	23.9	48.2	36.3	11.8
10,000	40.1	36.8	48.0	40.0	11.6

PEG-10,000/phosphate system. Although the results for the *exo*-PG are not so good, the PFs and yield recoveries obtained in the present work indicate that ATPS can be a good alternative for purifying pectinolytic enzymes.

3.2. Influence of NaCl

Table 5 shows the effect of the addition of 6% (w/v) of NaCl on the partition coefficients. The standard deviations for the *K*-values varied between 0.020 and 0.002 for protein, 0.071 and 0.001 for PE, 0.040 and 0.001 for PL, 0.220 and 0.023 for *exo*-PG and 0.110 and 0.001 for *endo*-PG.

In the systems containing sodium chloride the enzyme partition coefficients exhibit the same trend already observed in the systems without this salt, in most cases they decrease as the PEG molecular weight increases, attaining a minimum value for PEG-3350 or 8000 and then they increase slightly for PEGs of higher molecular weight. In most cases the addition of NaCl decrease the enzyme partition coefficients. The most remarkable exception to this behaviour was obtained for *exo*-PG, whose partition coefficient has it highest value for the system with PEG-400 and NaCl (5.35).

The addition of NaCl contributed to the increase of the protein yield recovery in the upper phase (Table 6). A similar trend was observed for the yield recovery of *exo*-PG, but the opposite behaviour predominated in the case of *endo*-PG and PE. The addition has a more pronounced effect upon the protein partition coefficient, increasing their values. As a consequence, in most cases the PFs decreased as sodium chloride was added to the system (Table 7). Lahore et al. (1995) also observed the same behaviour for the partition of acid protease from *M. bacilliforms* in systems PEG-potassium phosphate with sodium chloride.

Table 7
PF in the upper phase in systems PEG-phosphate with 6% (w/v) of NaCl at 4 °C and pH 7.0

PEG	*Endo*-PG	*Exo*-PG	PL	PE
400	0.94	1.29	1.33	0.98
600	1.22	1.18	1.99	1.21
1000	2.46	0.93	3.66	2.07
1450	4.65	1.48	6.55	4.35
3350	6.67	2.09	8.63	5.89
8000	2.62	2.03	4.08	3.05
10,000	3.60	3.16	4.14	3.45

Acknowledgements

The authors would like to thanks New Nordisk-Brazil for providing the Pectinex-3XL® (commercial enzyme). This investigation was supported by research grants of FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

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