

## Pectinases partitioning in aqueous two-phase systems: an integration of the systems poly(ethylene glycol)/crude dextran and poly(ethylene glycol)/ammonium sulphate

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(Received 4 November 2003)

*Abstract:* The partitioning of pectinases in the poly(ethylene glycol)4000/ammonium sulphate system was studied and also its application for enzymes extraction from the top phase of the poly(ethylene glycol)4000/crude dextran system. Almost complete one-sided partition of endo-pectinase and exo-pectinase to the bottom phase of the polymer/salt system was achieved at a tie-line length of 37.16 %. The concentration factors were 1.73 and 3.25, respectively. The highest total endo- and exo-pectinase yields (72.41 % and 69.46 %, respectively) were obtained by integration of the polymer/polymer system at a tie-line of 8.61 % and a high phase volume ratio and the polymer/salt system at a tie-line of 30.23 % and a low phase volume ratio. Integration of the partitioning at a high tie-line length in the polymer/polymer and a low tie-line length in the polymer/salt system resulted in a total concentration factor of 1.5 and a purification of 1.66 fold for exo-pectinase. The addition of phosphate to this integrated system improved the total concentration factor and purification fold of the activity to 1.73 and 2.14, respectively.

*Keywords:* aqueous two-phase system, partitioning, pectinases.

### INTRODUCTION

Pectinases are a group of enzymes that degrade pectic substances by different mechanisms. From a commercial point of view, pectinases produced by cultivation of fungi are of the greatest importance, finding wide application in the food industry.<sup>1</sup> Apart from these industrial applications, pectinases have biological importance in protoplast fusion technology and plant pathology.<sup>2</sup>

Generally, the separation and purification of enzymes from cultivation media constitutes a major part in the final economic calculation of the feasibility of their production. For this reason, a need exists for an efficient, effective and economic

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large-scale bioseparation technique to achieve high purity and high recovery, while maintaining the biological activity of the enzyme.

Aqueous two-phase systems (ATPSs), formed by mixing two, mutually incompatible polymers, or one polymer and an inorganic salt, are important for the separation and purification of enzymes, proteins and other substances in biological processes.<sup>3</sup> They allow process integration, as both separation and concentration of the target product are simultaneously achieved.<sup>4</sup> As ATPSs are easily scalable and are also able to hold high biomass loads in comparison with other separation techniques, their application for the recovery of enzymes from cultivation broths has attracted most interest so far.<sup>5,6</sup>

The most commonly used polymer/polymer system is composed of dextran and poly(ethylene glycol), but it is expensive for scaling up. This problem can be overcome by the use of non-fractionated crude dextran<sup>7,8</sup> or by the substitution of dextran by cheaper non-toxic polymers<sup>9,10</sup> or by recycling the poly(ethylene glycol).<sup>11</sup>

This paper presents the results of an investigation of an integrated approach to pectinases extraction in aqueous two-phase systems, by using crude dextran, on the one hand, and by investigating the possibilities for recovery of the poly(ethylene glycol), on the other. The partition of pectinases in poly(ethylene glycol)/ammonium sulphate ATPS was studied, and the its application for enzyme recovery from the top phase of a poly(ethylene glycol)/crude dextran two-phase system. The main factors affecting the enzyme partition behaviour, such as tie-line length and phase volume ratio, were evaluated, aiming at determining firstly – the optimal partition conditions for enzymes appearance in the bottom phase of the polymers/salt ATPS and secondly – the most favourable combination of integrated systems for enzymes recovery from the top phase of the polymer/polymer system to the bottom phase of the polymer/salt system, allowing recycling of the top phase polymer.

## EXPERIMENTAL

### *Commercial pectinase preparation*

In the partition studies in the poly(ethylene glycol)/ammonium sulphate ATPS, when examination of the influence of the tie-line length and phase volume ratio were being conducted, Vinoxym (Novo Nordisk, Denmark) was diluted 100 times in 10 mmol l<sup>-1</sup> acetate buffer pH 5.0 to make the basal enzyme solution (BES). In order to examine the partition behaviour of pectinases in the poly(ethylene glycol)/crude dextran ATPS, Vinoxym was diluted 100 times in 10 mmol l<sup>-1</sup> acetate buffer, pH 5.0, and in 0.3 mol l<sup>-1</sup> phosphate (KH<sub>2</sub>PO<sub>4</sub>-Na<sub>2</sub>HPO<sub>4</sub>) buffer, pH 7.0, to make the basal enzyme solutions.

### *Preparation of the ATPS systems*

The polymers used were polyethylene glycol 4000 (PEG) (MW 3,500–4,500, Merck, Germany) and crude dextran (DEX) (MW ≈ 300·10<sup>3</sup>). A cryde dextran solution of 10 % (w/w) contained approximately 0.5 % reducing sugars, as determined by the DNS-method,<sup>12</sup> with glucose as the standard.

Phase systems for the model system experiments were constructed according to the literature<sup>13</sup> by mixing thoroughly the required quantities of PEG and ammonium sulphate or DEX in the enzyme solutions, pre-equilibrated at room temperature, for 5 min at vortex. The total mass of two-phase system was 10 g. The two phases were allowed to separate (12 h) before sampling, and

then the upper phase was carefully removed with a pipette, leaving a small amount at the interface. The lower phase was then sampled through the interface. Samples of each phase were analysed for enzyme activity.

#### Enzyme assays

Endo-pectinase (endo-p) activity was determined by measuring the decrease of the specific viscosity of the reaction mixture.<sup>14</sup> One unit was defined as the amount of enzyme that reduced the initial specific viscosity of the reaction mixture by 20 % in 1 min. In order to avoid the influence of sample viscosities on the analytical procedures, suitable dilutions were made such that the initial viscosities of the reaction mixture, after addition of the basal enzyme solution of commercial pectinases or samples, were the same.

Exo-pectinase (exo-p) activity was measured according to the literature.<sup>15</sup> One unit was defined as the amount of enzyme that catalysed the formation of 1  $\mu\text{mol}$  of galacturonic acid per hour at pH 5.0.

The partition coefficients for endo-p and exo-p in the aqueous two-phase systems were defined as:

$$K = \frac{\text{activity}_{\text{top phase}}}{\text{activity}_{\text{bottom phase}}} \quad (1)$$

the yield in the top phase as:

$$Y_t (\%) = \frac{100}{\left(\frac{V_t}{V_b} K\right)^{-1} + 1} \quad (2)$$

and the yield in the bottom phase as:

$$Y_b (\%) = \frac{100}{\frac{V_t}{V_b} K + 1} \quad (3)$$

where  $V_t$  and  $V_b$  are the volumes of the top and bottom phase, respectively.

The concentration factor for the enzyme activities in the phases was defined as:

$$CF_{\text{phase}} = \frac{\text{activity}_{\text{phase}}}{\text{activity}_{\text{BES}}} \quad (4)$$

and the purification factor in the top and the bottom phase as:

$$PF_{\text{phase}} = \frac{\text{specific activity}_{\text{phase}}}{\text{specific activity}_{\text{BES}}} \quad (5)$$

The tie-line length was defined<sup>16</sup> as

$$\text{Tie-line length} = [(w_1^{\text{TOP}} - w_1^{\text{BOT}})^2 + (w_2^{\text{TOP}} - w_2^{\text{BOT}})^2]^{1/2} \quad (6)$$

where  $w_i^{\text{TOP}}$  and  $w_i^{\text{BOT}}$  represent the weight percentages of the phase-forming component  $i$  in the top and bottom phase, respectively.

The results are the mean value of at least three measurements of activity (the accuracy is considered to be  $\pm 5\%$ ) on a minimum of three replicas for every partition experimental point.

### Analytical methods

The protein concentration was determined according to Bradford.<sup>17</sup> The dextran content in the phases was determined in a polarimeter<sup>18</sup> (Perkin-Elmer) at 589 nm and the concentration of polyethylene glycol 4000 was measured as described elsewhere.<sup>19</sup> The concentration of ammonium sulphate was also determined.<sup>20</sup>

## RESULTS AND DISCUSSION

Increasing the tie-line length is reported to increase the hydrophobicity of the top phase in an ATPS and also the interfacial potential between the two phases.<sup>3</sup> To evaluate the effect of the tie-line length on pectinases partition in the PEG 4000/ammonium sulphate ATPS, a commercial preparation was diluted in acetate buffer. The concentrations of the phase constituents were chosen to ensure that the phase volume ratio amounted 1 in all experiments (Table I).

TABLE I. Effect of tie-line length<sup>a</sup> in the PEG 4000/ammonium sulphate ATPS on the partition coefficient and yield, concentration factor and purification fold in the bottom phase of endo-p and exo-p activities of a commercial preparation<sup>b</sup>

PEG %(w/w)	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> %(w/w)	Tie-line length/%	Endo-p				Exo-p			
			<i>K</i>	<i>Y<sub>b</sub></i> /%	<i>CF<sub>b</sub></i>	<i>PF<sub>b</sub></i>	<i>K</i>	<i>Y<sub>b</sub></i> /%	<i>CF<sub>b</sub></i>	<i>PF<sub>b</sub></i>
13	10.5	25.28	0.30	76.92	0.98	0.94	0.029	97.18	1.88	1.80
16	11.0	30.23	0.00	100	2.18	1.62	0.021	97.94	2.71	2.01
19	12.0	37.16	0.00	100	1.78	1.05	0.016	98.42	3.25	1.92

<sup>a</sup> The phase volume ratios were 1 in all experiments; <sup>b</sup> The commercial pectinase preparation was diluted 100 times in 0.1 mol l<sup>-1</sup> acetate buffer, pH 5.0, so the initial endo-p and exo-p activities of the basal enzyme solution were 20.83 U ml<sup>-1</sup> and 3220 U ml<sup>-1</sup>, respectively, with a protein concentration 0.043 mg ml<sup>-1</sup>

Increasing the tie-line length favoured partition of both endo- and exo-pectinase to the bottom phase, which resulted in an increase in the yields of enzyme activities in that phase and, consequently, a decrease in the partition coefficients. A similar trend was observed in the system PEG 8000/ammonium sulphate for endo-polygalacturonase from *Klayveromyces marxianus* at tie-lines below 30%.<sup>11</sup> So-called one-sided partition of endo-p was achieved for the examined tie-lines when the length was over 30%, followed by maximal enzyme yield in the salt rich phase. An increase in the tie-line length was also followed by an increase in the concentration factor of pectinases in the bottom phase, but the most appropriate conditions for selective partitioning of the proteins, corresponding to pectinase activities, were with a tie-line of 30.23%, under which the highest purification folds of 1.62 and 2.01 were obtained for endo-p and exo-p, respectively.

These results indicated the possible application of the system of interest for the recovery of enzymes in the bottom phase, accompanied by their consequential concentra-

tion and purification. Hence, in the following experiments, the effect of the phase volume ratio on the partition parameters was examined in order to find their optimal combination, as it is known that for high yields both  $K$  and  $V_t/V_b$  have to be optimised.<sup>21</sup>

Increasing the phase volume ratio along a tie-line the length of which was 25.28 % caused a decrease in the partition coefficient and in the yield in the bottom phase of both pectinase activities (Table II). These results are in agreement with results obtained for the partition of yeast's endo-polygalacturonase at higher phase volume ratios in the system PEG 8000/ammonium sulphate.<sup>11</sup> On the other hand, increasing the volume of the top phase at the expense of the bottom phase increased the concentration factor of endo-p and exo-p in the bottom phase by 2.5 and 2.1 times, respectively, along with increasing phase volume ratio from 0.2 to 2.83. However, the best purification of both enzymes was achieved when the volumes of the phases were equal.

TABLE II. The effect of the phase volume ratio along a single tie-line<sup>a</sup> in the PEG 4000/ammonium sulphate ATPS on the partition coefficient and yield, concentration factor and purification fold in the bottom phase of endo-p and exo-p activities of a commercial preparation<sup>b</sup>

PEG %(w/w)	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> %(w/w)	$V_t/V_b$	Endo-p				Exo-p			
			$K$	$Y_b/\%$	$CF_b$	$PF_b$	$K$	$Y_b/\%$	$CF_b$	$PF_b$
5.0	14.0	0.20	0.59	89.44	0.80	0.37	0.042	99.17	1.03	0.47
7.5	13.0	0.34	0.43	87.24	0.77	0.48	0.020	99.32	2.20	1.35
13.0	10.5	1.00	0.30	76.92	0.98	0.94	0.029	97.18	1.88	1.80
17.5	8.5	2.83	0.14	71.62	2.00	0.72	0.012	96.72	2.14	0.77

<sup>a</sup> The tie-line length was 25.28 %; <sup>b</sup> Commercial pectinase preparation as for Table I

The obtained results indicate a possible application of the PEG 4000/ammonium sulphate ATPS for achieving one-sided endo- and exo-pectinase partitioning, expressed by a maximal bottom phase yield, and an enzyme free top phase suitable for polymer recycling, at tie-lines above 30 % and at phase volume ratio below 0.3. Also, if concentration of the enzyme is required the tie-line length should be a long one, but the phase volume ratio should be as high as possible.

Considering these conclusions, the next experiments were conducted to examine the integration of two kinds of aqueous two-phase systems: polyethylene glycol 4000/crude dextran, which is reported to be suitable for extractive cultivation for pectinase production,<sup>22-24</sup> for enzyme partitioning to the top phase, and polyethylene glycol 4000/ammonium sulphate for subsequent enzyme extraction to the bottom phase, opening the possibility for recycling of the PEG from the top phase. The first series of experiments were conducted using a commercial pectinase preparation diluted in acetate buffer, pH 5.0, at different combinations of tie-line lengths and phase volume ratios (Table III). The second system was prepared by adding solid salt to the top phase collected from the first system.

TABLE III. Yields, concentration factors and purification folds of endo-p and exo-p activities from commercial pectinase preparation<sup>a</sup>, expressed by partition steps, in different combinations of integrated aqueous two-phase systems

Partit. step <sup>b</sup>	ATPS	$V_t/V_b$	TLL/%	Phase	Endo-p			Exo-p		
					Y/%	CF	PF	Y/%	CF	PF
1	PEG/DEX	1.30	7.13	Top <sup>1</sup>	58.94	0.51	0.65	48.56	0.61	0.78
2	Top <sup>1</sup> /(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.19	25.50	Bottom	100	1.25	1.12	99.02	0.83	0.74
1	PEG/DEX	2.92	8.61	Top <sup>2</sup>	72.41	0.65	0.58	69.46	0.94	0.85
2	Top <sup>2</sup> /(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.18	30.23	Bottom	100	1.02	1.23	100	1.30	1.58
1	PEG/DEX	1.44	9.45	Top <sup>3</sup>	49.03	0.56	1.23	53.46	0.66	1.44
2	Top <sup>3</sup> /(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.20	30.23	Bottom	100	1.64	0.69	97.24	1.07	0.45
1	PEG/DEX	1.00	9.81	Top <sup>4</sup>	60.00	0.53	0.38	57.63	0.88	0.63
2	Top <sup>4</sup> /(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.19	27.40	Bottom	100	1.15	1.76	99.58	1.72	2.64

<sup>a</sup>commercial pectinase preparation as in Table I 0.043 mg ml<sup>-1</sup>; <sup>b</sup>The second system was prepared by adding solid salt to the top phase collected from the first system

All the examined combinations of integrated two-phase systems resulted in 100 % extraction of endo-p from the top phase, generated in the first partition step, irrespective of the working tie-line length and phase volume ratio. The highest total yields of endo-p and exo-p (72.41 % and 69.46 %, respectively), calculated as the result of two sequential partition steps, were achieved in the polymer/polymer system at a tie-line length of 8.61 % and a high phase volume ratio (2.92) in combination with a polymer/salt system at a working tie-line length of 30.23 % and  $V_t/V_b = 0.18$ . Considering the concentration factor of endo-p, the highest total value, 0.92, calculated as the result of two sequential partition steps, was obtained in the third examined system which integrated a first partition step using an intermediate tie-line length and intermediate phase volume ratio with a higher tie-line length and, contrary to the previous results (Table II), a high bottom phase volume in the second partition step. Also, in the same integrated system, the exo-p activity was about 1.5 times higher after double partition in the ATPSs than in the basal enzyme solution. These results might be explained by new partition conditions in the polymer/salt system, because of the small, but definite presence of dextran in the second system, originating from the first one. They show that the idea of examining the optimal combination of integrated aqueous two-phase systems was justified. As for the purification of exo-p, the combination of a long tie-line in the first and a short one in the second ATPS, with low phase volume ratios, gave the highest achieved total purification fold of 1.66.

The next experiments were conducted in the presence of phosphate buffer, 0.3 mol l<sup>-1</sup>, pH 7.0, for the purpose of improving the partition parameters in the first aqueous two-phase system, as was reported in the literature.<sup>18,24,25</sup> Two systems

were studied (Table IV), which corresponded to the lowest and to the highest TLL in previously examined PEG/DEX aqueous two-phase systems.

Generally, the presence of phosphate and the higher pH of the system improved the partition coefficients for both pectinase activities in the first partition step (data not shown), but, simultaneously, the phase volume ratio was diminished, so that the combined effect was almost zero or even a decrease in the top phase yield for the two examined tie-line lengths in the polymer/polymer system compared with systems without phosphate (Tables III and IV). Also, improved concentration factors and, especially, purification folds for both endo-p and exo-p were obtained in the top phases, irrespective of the system TLL in the phosphate buffer.

TABLE IV. Yields, concentration factors and purification folds of endo-p and exo-p activities from commercial pectinase preparation<sup>a</sup>, expressed by partition steps, in different combinations of integrated aqueous two-phase systems

Partit. step <sup>b</sup>	ATPS	$V_t/V_b$	TLL/%	Phase	Endo-p			Exo-p		
					Y/%	CF	PF	Y/%	CF	PF
1	PEG/DEX	0.64	7.13	Top <sup>1</sup>	46.59	0.74	0.94	49.86	0.83	1.06
2	Top <sup>1</sup> /(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.17	25.50	Bottom	100	1.19	1.03	98.78	0.83	0.72
1	PEG/DEX	0.79	9.81	Top <sup>2</sup>	61.95	0.77	0.97	55.69	1.53	1.93
2	Top <sup>2</sup> /(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.15	27.40	Bottom	100	0.80	0.78	98.13	1.13	1.11

<sup>a</sup>Commercial pectinase preparation was diluted 100 times in 0.3 mol l<sup>-1</sup> (KH<sub>2</sub>PO<sub>4</sub>-Na<sub>2</sub>HPO<sub>4</sub>) phosphate buffer, pH 7.0, so the initial endo-p and exo-p activities of the basal enzyme solution were 7.94 U ml<sup>-1</sup> and 2440 U ml<sup>-1</sup>, respectively, with a protein concentration 0.067 mg ml<sup>-1</sup>; <sup>b</sup>The second system was prepared by adding solid salt to the top phase collected from the first system

Phosphate did not influence the yield of endo-pectinase in the polymer/salt system, but decreased it in the case of exo-p. However, the presence of two salts in the ATPS, phosphate and sulphate, had a diminishing effect on the concentrating of the enzymes in the bottom phase, so the overall concentration factors for the enzymes in the first combination of two-phase systems were lower than in the system containing only ammonium sulphate. These results may be explained by the detrimental effect of the salt combination, as it is known that it is the ratio between the different ions that mainly influences the partition phenomena.<sup>26</sup> In the second integration of the systems, overall the concentration factor for exo-p was slightly improved by about 15 %. On the other hand, the presence of phosphate salts at the higher pH improved the purification of the enzymes in both integrated systems, which resulted in an improved specific exo-p activity in the bottom phase of the second integration system by more than 2 fold.

From the results obtained in this study, it can be concluded that the partition parameters of endo-p and exo-p in an aqueous two-phase system composed of poly(ethylene glycol) 4000 and ammonium sulphate indicate the application of

this ATPS for complete enzyme recovery from the top phase of the poly(ethylene glycol)4000/crude dextran system, opening the possibility for top phase polymer recycling. Furthermore, considering the fact that much higher values of partition parameters for both pectinases were achieved during cultivation of the fungus *Polyporus squamosus* in PEG 4000/crude dextran aqueous two-phase system<sup>22-24</sup> than in the model system, better results of the application of such an integrated approach might be expected.

*Acknowledgements:* This work was supported by grant number 1394 from Ministry of Science, Technology and Development, Republic of Serbia.

#### ИЗВОД

### РАСПОДЕЛА ПЕКТИНАЗА У ВОДЕНИМ ДВОФАЗНИМ СИСТЕМИМА: ИНТЕГРАЦИЈА СИСТЕМА ПОЛИЕТИЛЕН ГЛИКОЛ/СИРОВИ ДЕКСТРАН И ПОЛИЕТИЛЕН ГЛИКОЛ/АМОНИЈУМ СУЛФАТ

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Испитивана је расподела пектиназа у систему полиетилен гликол 4000/амонијум сулфат, као и примена у екстракцији ензима из горње фазе система полиетилен гликол 4000/сирови декстран. Добијена је готово потпуна расподела ендо-пектиназе и егзо-пектиназе у доњу фазу система полимер/со при дужини везне линије од 37,16 %, уз факторе концентрисања 1,73 и 3,25, редом. Највећи укупни приноси ендо- и егзо-пектиназе добијени су интеграцијом система полимер/полимер, при везној линији 30,23 % и ниском запреминском односу фазе, и износили су 72,41 % и 69,46 %, редом. Интеграцијом расподела на великој дужини везне линије у систему полимер/полимер и малој дужини везне линије у систему полимер/со добијен је укупни фактор концентрисања 1,5 и степен прецизности 1,66 за егзо-пектиназу, а додаток фосфата у овако интегрисани систем повећао је вредности ових параметара на 1,73 и 2,14, редом.

(Примљено 4. новембра 2003)

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