ANNALS OF THE FACULTY OF ENGINEERING HUNEDOARA
2003, Tome I, Fascicole 3

PARTITIONING OF PECTINASES IN AQUEOUS TWO-PHASE SYSTEM POLYETHYLENE GLYCOL/DEXTRAN 500,000

Senka MAĐAREV, Mirjana ANTOV, Draginja PERIČIN

UNIVERSITY OF NOVI SAD, FACULTY OF TECHNOLOGY,
SERBIA AND MONTENEGRO

ABSTRACT/SUMMARY

Partitioning of pectinases, enzymes of great industrial importance, in aqueous two-phase system composed of polyethylene glycol and dextran, was studied. In system 7.5% (w/w) polyethylene glycol 1500/ 7.5% (w/w) dextran 500,000 the partition coefficient of endo-pectinase and the top phase yield was 0.88 and 42.55%, respectively. In the same system the partition coefficient of exo-pectinase was 1.28 what was followed by the top phase yield 52%.
In system 7.5% (w/w) polyethylene glycol 4000/ 7.5% (w/w) dextran 500,000 obtained partition coefficients of endo-pectinase and exo-pectinase were higher and amounted 0.95 and 0.96, respectively. That caused both higher top phase yield for enzyme activities (about 65%) and phase volume ratio in comparison to system containing polyethylene glycol 1500.

KEY WORDS:
aqueous two-phase system, pectinases

1. Introduction

Aqueous two-phase system provides a technically simple, easily scalable, energy efficient and mild separation technique for recovery of biomolecules (Albertsson, 1986). When applied in biotechnological processes they allow process integration, as simultaneous separation and concentration of the target product are achieving. Because ATPSs are easily scalable and are also able to hold high biomass load in comparison with other separation techniques, their application for recovery of enzymes from cultivation broth has attracted most interest so far (Antov et al., 2001).

Aqueous two-phase system is formed as a result of mixing aqueous solutions of two, mutually incompatible, water-soluble polymers or one polymer and salt in high concentration. The basis of separation in ATPS is the selective distribution of substances between two phases, governed by a number of parameters relating to the properties of the phase system and the substance, and the interaction between them (Albertsson, 1986).

The most commonly used polymer/ polymer system is composed of dextran and polyethylene glycol because those polymers are not toxic,

there are biocompatible and environmental friendly and they are even used in human drug formulations. In such systems molecular weight of polymers might have an influence on partition of target substance (Kula, 1979).

Pectinases are a group of extracellular enzymes that degrade pectic substances by different mechanisms and, from a commercial point of view, are of the great importance, having wide application in the food industry (Fogarty and Kelly, 1983).

The aim of the study was to examine the influence of molecular weight of polyethylene glycol as well as the concentration of phase forming polymers on the partition coefficient and the top phase yield of endo-pectinase and exo-pectinase activities in ATPS, composed of polyethylene glycol and dextran 500,000. Also, the possible application of ATPS for extractive bioconversion of pectin was investigated.

2. Material and Methods

Commercial pectinase preparation

In the partition studies Vinozym (Novozymes, Denmark) was diluted 100 times in 10 mmol l^{-1} acetate buffer pH 5.0 to make the basal enzyme solution.

Preparation of ATPS

The polymers used were polyethylene glycol 1500 (PEG 1500) and 4000 (PEG 4000) (Merck, Germany) and fractionated dextran 500,000 (DEX) (Fluka, Switzerland).

Phase systems were constructed, according to literature (Hotha and Banik, 1997), by mixing thoroughly the required quantities of polymers in the enzyme solutions, equilibrated at room temperature, for 5 minutes at vortex. The total mass of the two-phase system was 10 g. The two phases were allowed to separate (12 hours) 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 activities or reducing sugars.

The partition coefficients in the aqueous two-phase systems were defined as

$$K = \frac{\text{activity (concentration)}_{\text{topphase}}}{\text{activity (concentration)}_{\text{bottom phase}}} \qquad [1]$$

yield in the top phase as

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

and yield in the bottom phase as

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

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

The tie-line length (TLL) was defined (Furuya et al., 1996) as

$$\text{Tie} - \text{line length} = \left[\left(w_1^{TOP} - w_1^{BOT}\right)^2 + \left(w_2^{TOP} - w_2^{BOT}\right)^2\right]^{1/2} \qquad [4]$$

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

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

Enzyme assays

Endo-pectinase (endo-p) activity was determined by measuring the decrease of the specific viscosity of the reaction mixture (Peričin et al., 1992). 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 analytical procedures, suitable dilutions were made such that the initial viscosities of reaction mixture, when basal enzyme solution of commercial pectinases was added, had the same value.

Exo-pectinase (exo-p) activity was measured according to literature (Aguilar and Huitron, 1990). One unit was defined as the amount of enzyme that catalysed the formation of 1μmol of galacturonic acid per hour at pH 5.0.

Batch hydrolysis of pectin

Aqueous two-phase system for batch hydrolysis contained 12.5% (w/w) PEGS 1500/ 3% (w/w) DEX/ 1% (w/w) apple pectin (Red Ribbon Pure, Obipektin, Switzerland). Hydrolysis have been performed for 3 hours on magnetic stirrer at room temperature, then phases were separated in bench-scale centrifuge (3 min at 3000 rpm) and analysed for reducing sugars.

Analytical methods

Dextran content in the phases was determined, according to the literature (Andersson et al., 1985), in a polarimeter (Perkin-Elmer) at 589 nm. Concentration of polyethylene glycol was measured as described elsewhere (Skoog, 1979).

Reducing sugars were determined by DNS method (Miller, 1969) with galacturonic acid as standard.

RESULTS AND DISCUSSION

The influence of molecular mass of polyethylene glycol on the partitioning of endo and exo-pectinase activities were examined in

aqueous two-phase systems containing equal concentration of polymers (Table 1). Higher molecular mass of the top phase polymer yielded system with greater volume of the top phase and consequently higher phase volume ratio. It also influenced the partition coefficients for enzymes but in the different manner. Presence of polyethylene glycol 4000 favoured partition of endo-p activity to the top phase of the system in comparison to PEG 1500. On the contrary, when polyethylene glycol of lower molecular mass was present higher partition coefficient of exo-pectinase was achieved.

TABLE 1. THE INFLUENCE OF MOLECULAR MASS OF POLYETHYLENE GLYCOL ON THE PARTITION PARAMETERS OF ENDO AND EXO-PECTINASE ACTIVITIES IN AQUEOUS TWO-PHASE SYSTEMS

ATPS % (w/w)	V_t/V_b	Endo-p activity		Exo-p activity	
		K	Y_t (%)	K	Y_t (%)
7.5 PEG 1500 7.5 DEX	0.84	0.88	42.55	1.28	51.86
7.5 PEG 4000 7.5 DEX	1.94	0.95	64.77	0.96	65.01

Higher yield of endo-pectinase was obtained in system with PEG 4000 as the consequence of both favourable partition coefficient and phase volume ratio. As for exo-p activity, higher top phase yield was also attained in system containing polyethylene glycol of higher molecular mass in spite of the fact that lower partition coefficient was obtained. It is in agreement that both favourable partition coefficient and phase volume ratio are important for yield in ATPS (Antov and Peričin, 2001).

In aqueous two-phase system composed of polyethylene glycol 1500 and dextran 500,000 the influence of concentration of polymers on the partition parameters was examined (Table 2). Composition of ATPS was varied along the tie-line whose length was 20 % (w/w). It is known that two-phase systems with overall composition represented by one of the tie-lines yielded phases with identical composition but different volumes.

TABLE 2. THE INFLUENCE OF ATPS COMPOSITION ALONG THE TIE-LINE LENGTH 20% (W/W) ON THE PARTITION PARAMETERS OF ENDO AND EXO-PECTINASE ACTIVITIES IN SYSTEM PEG 1500/ DEX

ATPS % (w/w)	V_t/V_b	Endo-p activity		Exo-p activity	
		K	Y_t (%)	K	Y_t (%)
6.5 PEG 12.5 DEX	0.38	0.84	24.2	2.25	46.1
7.5 PEG 10.5 DEX	0.78	1.00	43.8	0.74	36.6
9.0 PEG 8.5 DEX	0.88	1.89	62.4	1.04	47.8
10.5 PEG 6.0 DEX	2.39	0.87	67.5	0.97	69.9
12.5 PEG 3.0 DEX	5.55	0.81	81.8	0.29	61.7

The highest partition coefficient of endo-p activity was achieved in system composed of 9% (w/w) PEG 1500 and 8.5% (w/w) DEX and amounted 1.89, but the highest yield in the top phase, over 80%, was obtained in the system with the highest examined phase volume ratio.

System, composed of 6.5% (w/w) PEG 1500 and 12.5 % (w/w) DEX, yielding the lowest examined phase volume ratio, was the most favourable

for the partition of exo-p activity to the top phase and consequently the highest partition coefficient was attained. The highest top phase yield, about 70%, was obtained in system with phase volume ratio 2.39, while further increase in V_t/V_b decreased it through dramatic decrease in partition coefficient.

Aqueous two-phase system 12.5% (w/w) PEG 1500/ 3% (w/w) DEX was preliminarily examined as possible medium for pectin hydrolysis. It is known that by action of pectinase pectin can be converted into oligogalacturonides whose act as prebiotic fibbers (Lang and Dornenburg, 2000). Batch bioconversion was performed and partition of degradation products, measured as reducing sugars (expressed as galacturonic acid), was evaluated (Table 3).

TABLE 3. PARTITION PARAMETERS OF BATCH HYDROLYSIS OF PECTIN IN 12.5% (W/W) PEG 1500/ 3% (W/W) DEX AQUEOUS TWO-PHASE SYSTEM

Batch hydrolysis	V_t/V_b	Reducing sugars		
		K	Y_t (%)	Y_b (%)
Before	6.15	0.40	91	9
After 3 hours	5.33	0.01	69	31

Before hydrolysis majority of pectin substances, over 90%, were partitioned to the top phase, where were located more than 80% of endo-p and 60% of exo-p activities (Table 2). After 3 hours of hydrolysis, conditions regarding phase volume ratio and partition parameters were changed for the benefit of partition of degradation products to the bottom phase, i.e. the bottom phase yield amounted about 30%.

Obtained results suggested that the use of aqueous two-phase system, composed of polyethylene glycol and dextran, for the extractive bioconversion of pectin is possible. Favourable circumstance is that majority of pectinase activities are partitioned to the top phase, while products of pectin bioconversion, considering their partition coefficient, had greater affinity to the bottom phase, which opens the possibility of recycling (reusing) the enzyme containing top phase. For future work, conditions regarding composition of aqueous two-phase system, phase volume ratio etc. need to be optimized.

Acknowledgements

This work was supported by grant number 1394 from Ministry of Science and Environmental Protection, Republic of Serbia.

REFERENCES

[1.] AGUILAR, G., HUITRON, C. (1990) Constitutive exo-pectinase produced by *Aspergillus* sp. CH-Y-1043 on different carbon source. *Biotechnology Letters* 12, 655-660.

[2.] ALBERTSSON, P-A. (1986) Partition of Cell Particles and Macromolecules, Wiley & Sons, New York.

[3.] ANDERSSON, E., JOHANSSON, A.C., & HAHN-HAGERDAL, B. (1985) α-Amylase production in aqueous two-phase systems with *Bacillus subtilis*. *Enzyme and Microbial Technology* 7, 333-338.

[4.] ANTOV, M., PERIČIN, D (2001) Production of pectinases by *Polyporus squamosus* in aqueous two-phase system. *Enzyme and Microbial Technology* 28, 467-472.
[5.] ANTOV, M., PERIČIN, D., DIMIĆ, G. (2001) Cultivation of *Polyporus squamosus* for pectinase production in aqueous two-phase system containing sugar beet extraction waste. *Journal of Biotechnology* 91, 83-87.
[6.] FOGARTY, W., KELLY, C. (1983) Pectic enzymes. In: Microbial Enzyme and Biotechnology (Fogarty, W., editor). Applied Science Publishers, London, 131-182.
[7.] FURUYA, T., YAMADA, S., ZHU, J., YAMAGUCHI, Y., IWAI, Y., & ARAI, Y. (1996) Measurement and correlation of liquid-liquid equillibria and partition coefficients of hydrolytic enzymes for DEX T500 + PEG 20000 + water aqueous two-phase system at 20^{o}C. *Fluid Phase Equillibria* 125, 89-102.
[8.] HOTHA, S., BANIK, R.M. (1997) Production of alkaline protease by *Bacillus thuringiensis* H14 in aqueous two-phase systems. *Journal of Chemical Technology and Biotechnology* 69, 5-10.
[9.] KULA, M-R. (1979) Extraction and purification of enzymes using aqueous two-phase systems. *Applied Biochemistry and Bioengineering* 2, 71-95.
[10.] LANG, C., DORNENBURG, H. (2000) Perspectives in the biological function and the technological application of polygalacturonases. *Applied Microbiology and Biotechnology* 53, 366-375.
[11.] Miller, G.L. (1959) Use of dinitrosalicilyc acid reagent for determination of reducing sugar. *Analytical Chemistry* 31, 426-428.
[12.] PERIČIN, D., KEVREŠAN, S., BANKA, L., ANTOV, M., ŠKRINJAR, M. (1992) Separation of pectinolytic complex produced by *Polyporus squamosus* in submerged culture. *Biotechnology Letters* 14, 127-130.
[13.] SKOOG, B. (1979) Determination of polyethylene glycols 4000 and 6000 in plasma protein preparations. *Vox Sanguni* 37, 345-349.