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ORIGINAL ARTICLE

Purification of alkaline protease from chicken intestine by aqueous two phase system of polyethylene glycol and sodium citrate

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Abstract An aqueous two phase system of polyethylene glycol (PEG) and salts was evaluated for separation and purification of alkaline proteases from chicken intestine. Among the different salts evaluated potassium phosphate and sodium citrate gave higher enzyme yield (73.5% and 69.7% respectively) and enzyme purification (5.3 and 7.4 fold) in PEG rich upper phase. Increase in concentration of sodium citrate in the system resulted in reduction in enzyme yield and enzyme purification factor, with 15% salt showing highest enzyme yield (59.8%) and purification (6.7 fold). Initial protein concentration in the system did not show any specific trend on the partition behavior of the enzyme. The temperature at which the system is incubated did not show any significant ($p \geq 0.05$) effect on enzyme partition and purification. Increasing the PEG concentration in the system from 15 to 25% resulted in reduction in enzyme yield from 53.7 to 21.9% and enzyme purification from 5 fold to 1.4 fold in PEG rich upper phase. pH also had a significant ($p < 0.05$) effect on the partition of the enzyme to the upper phase with highest purification (3.4 fold) at pH 9.0 and higher enzyme yield (46.2%) at pH 10.

Keywords Chicken intestine · Alkaline protease · Aqueous two-phase system

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Introduction

Partitioning in an aqueous two phase system (ATPS) is a selective method used for purification of biomolecules by mixing two aqueous solutions of incompatible substances, such as polyethylene glycol (PEG) and dextran or PEG and salt above a critical concentration to form a liquid-liquid phase separation (Albertsson 1986; Diamond and Hsu 1992; Raghavarao et al. 1995). Solutions containing bio molecules when mixed to this type of two phase system, gets partitioned unequally between these two incompatible phases. The most common systems in use are polyethylene glycol (PEG)/dextran and PEG/salt systems (Oliveira et al. 2002; Su and Chiang 2006).

Industries desire ATPS, since it is less time consuming with high enzyme yield and purity (Andersson et al. 1985; Tanuja et al. 1997). ATPS also remove contaminants such as nucleic acids and unwanted proteins (Klomklao et al. 2005). ATPS is sometimes used as a potential primary purification technique to be followed by other selective final purification steps such as electrophoresis, chromatography etc. The differential partition in ATPS is governed by factors such as Van der Walls, hydrophobic, hydrogen bond and ionic interactions between the biomolecules and the surrounding phase (Su and Chiang 2006). Biomolecule partition is influenced by concentration and molecular weight of phase forming polymers such as PEG, concentration and type of added salt, temperature and pH of the system (Ufuk 2000). Partitioning also depends on their molecular weight, surface hydrophobicity, specific bonding site, surface charge and shape of the biomolecule to be separated (Francot et al. 1996; Han and Lee 1997). Employing ATPS in the industrial scale in downstream processing has been focused on the extraction, separation and concentration of a variety of bio molecules particularly

enzymes such as amylase (Oliveira et al. 2002), xylanase (Gaikaiwari et al. 1996), amyloglucosidase (Tanuja et al. 1997), pectinase (Antov 2004) and proteases (Oliveira et al. 2002; Spelzini et al. 2005; Tubio et al. 2007; Ravindra Babu et al. 2008). Reports on partition behavior characteristics of proteases of animal origin using ATPS are available (Spelzini et al. 2005; Reh et al. 2007; Tubio et al. 2007).

Proteases constitute one of the most important enzymes that are now used in a variety of industries such as detergent, food, pharmaceutical, leather and silk industries (Raju et al. 1997; Joo et al. 2001). Meat, fish and poultry processing industries generates large quantities of solid and liquid wastes such as heads, bones, intestines etc, These wastes / by-products are rich in bioactive molecules such as proteins, lipids, enzymes, heparin etc. (Shahidi and Kamil 2002; Warda et al. 2003; El-Beltagy et al. 2004). Poultry intestine which accounts for more than 20% of the poultry by-products, is rich in proteases and their level is comparable to that in organ tissues such as liver and spleen (Jamdar and Harikumar 2005). Reports on the recovery of bioactive molecules from poultry by-products such as intestine are seldom available. Further, application of ATPS for purification of proteases from poultry origin has not been attempted so far. Chicken intestinal proteases have been used to prepare protein hydrolysates from leather fleshings for use in animal feed formulations (Raju et al. 1997).

Against this background, in this study we report the purification of alkaline proteases from chicken intestine using ATPS. The study involved effect of different salts, salt concentration, protein concentration, pH, temperature and concentration of PEG on the partitioning behavior of chicken intestine protein and alkaline proteases in aqueous two phase system.

Materials and methods

Chicken intestine was obtained from the local market immediately after slaughtering the bird. The Polyethylene glycol (PEG) of molecular weight 6000 Daltons was obtained from Merck. All the salt and other chemicals were of analytical grade. Stock solutions of PEG and salts were prepared at 60% concentration and used for making the aqueous two phase system.

Preparation of crude enzyme extract About 50 gm of the minced chicken intestine was homogenised with 500 ml of cold ion free double distilled water for about 2–4 min at 3000–4000 rpm. The homogenised sample was centrifuged at 4°C for 10–12 min at 5000–6000 rpm. After centrifugation the supernatant was collected, filtered using Whatman No. 41 filter paper and made up to 500 ml and designated as crude enzyme extract (CE).

Determination of protein concentration and proteolytic activity The protein content in the extracts was determined by the method of Lowry et al. (Lowry et al. 1951) and calculated based on BSA standard curve. The activity of crude enzyme extract was determined at pH 8 (0.1 M phosphate buffer) using 1% casein as substrate. The assay mixture contained 1.25 ml buffer, 0.5 ml substrate and 0.25 ml enzyme extract. The assay mixture was incubated at 37°C for 30 min and the enzyme reaction was stopped by adding 3 ml of 5% TCA. For control TCA was added before adding the enzyme extract. The TCA extract was obtained by filtering the assay mixture using Whatman No. 1 filter paper. The TCA soluble peptides in the TCA extract was determined by the method of Lowry et al. (Lowry et al. 1951) and represented as tyrosine equivalents by referring to tyrosine standard curve. The specific activity of enzyme was expressed as μg tyrosine released per mg protein per min.

Aqueous two phase extraction ATPS was prepared in 15 ml centrifuge tubes by adding the appropriate quantities of stock solutions of PEG and salt (quantity determined from phase diagram) and volume was made up to 10 ml with crude extract and distilled water. To study the effect of different salts on purification of alkaline protease by ATPS, ammonium sulphate, magnesium sulphate, potassium phosphate (1:1.5 of mono- and dibasic salt), sodium phosphate (monobasic), sodium citrate, sodium sulphate and sodium carbonate were evaluated by maintaining the total level of PEG and salt in the system was set at 35% and the total volume was made up to with crude extract. To study the effect of salt concentration ATPS were prepared with PEG and salts- potassium phosphate and sodium citrate. The level of PEG was kept at a constant level of 20% and the salt content used was 15, 20 and 25%. 2.5 ml of crude extract was added to the system and wherever required the volume was made up to 10 ml with distilled water. As sodium citrate was found to be more suitable for purification of chicken intestine alkaline proteases by ATPS, the effect of protein concentration was evaluated using an ATPS consisting of PEG and sodium citrate. The level of PEG and salt was kept constant at 20 and 15% respectively. Crude enzyme extract was added to have different protein concentrations followed by distilled water to make up the volume, so that the protein concentration in system was 1.75, 1.5, 1.25, 1.0, 0.5 mg/ml system. The effect of temperature on the ATPS consisting of PEG and sodium citrate was evaluated by keeping the PEG and salt level constant at 20 and 15%, respectively. 2.5 ml of crude enzyme extract was added to the PEG-salt system, volume was made up to with distilled water and the tubes were incubated at 20°C, room temperature (RT), 37 and 45°C. The effect of different PEG concentration on the ATPS was studied by keeping the concentration of crude enzyme

extract (2.5 ml) and sodium citrate (15%) as constant, and varying the PEG concentrations (15, 20, 25% in the total mix). To study the effect of varying pH, ATPS was formed with 20% PEG, 15% sodium citrate. The volume of the crude extract was kept at 2.5 ml and the total volume of the system was made up to 10 ml with distilled water after adjusting the pH to 7, 8, 9 and 10 by adding different volumes of 0.1 M HCl or 1 M NaOH.

In all the experiments the contents of the centrifuge tubes were mixed thoroughly using a cyclo-mixer and were allowed to stand over night for complete separation of the phases. After clear separation of the phases, volumes of the separated phases- top layer, bottom layer and interface (if seen) is noted down. The top phase was carefully separated by using a Pasteur pipette and the interface (if any) was discarded. Aliquots from each phase were then taken for protein estimation and enzyme assay.

Determination of volume ratio, partition coefficient, yield and enzyme purification Volume ratio (VR) is defined as the ratio of the volume of the top phase to that of the bottom phase formed in the ATPS system of PEG and salt, i.e.,

$$\mathrm{VR} = \frac{\text{Volume of top phase(Vt)}}{\text{Volume of the bottom phase (Vb)}}$$

The bio-molecule *partition coefficient*, K, defined as the ratio of the bio-molecule concentration in the top phase to that in the bottom phase, is used to quantify the bio-molecule partition behavior, i.e.,

$$\mathrm{K} = \frac{[\mathrm{P}]_{\mathrm{top}}}{[\mathrm{P}]_{\mathrm{bottom}}}$$

where, $[P]_{top}$ and $[P]_{bottom}$ are macromolecule concentrations (protein content and specific activity of enzyme) in the top and bottom phase respectively.

The yield (Y) was calculated as follows

$$\text{Yield of protein}(\%) = \frac{100 \times (\mathrm{P_{t\,or\,b}} \times \mathrm{V_{t\,or\,b}})}{\mathrm{P_{CE}} \times \mathrm{V_{CE}}}$$

where, P_{CE} and $P_{t\ or\ b}$ are protein contents (mg/ml) in crude extract and top or bottom phase respectively , V_{CE} and $V_{t\ or\ b}$ are volume of crude extract and top or bottom phase respectively.

$$\text{Yield of enzyme}(\%) = \frac{100 \times (\mathrm{P_{t\,or\,b}} \times \mathrm{E_{t\,or\,b}} \times \mathrm{V_{t\,or\,b}})}{\mathrm{P_{CE}} \times \mathrm{E_{CE}} \times \mathrm{V_{CE}}}$$

where, P_{CE} and $P_{t\ or\ b}$ are protein contents (mg/ml) in crude extract and top or bottom phase respectively, E_{CE} and $E_{t\ or\ b}$ are enzyme activities (Specific activity) in crude extract and top or bottom phase respectively, V_{CE} and $V_{t\ or\ b}$ are volumes of crude extract and top or bottom phase respectively.

Enzyme purification factor is defined as the ratio of enzyme activity (specific activity) in top or bottom phase to that of crude extract

$$\mathrm{PF} = \frac{\text{Enzyme activity top or bottom}}{\text{Enzyme activity in crude extract}}$$

Statistical analysis All the experiments were repeated three times and the mean was determined. The statistical differences between treatments was analyzed by using ANOVA and mean separation was accomplished by Duncan's multiple range test using STATISTICA (Statsoft 1999).

Results and discussion

Effect of different salts The type of salt used in the ATPS had a significant ($p \leq 0.05$) effect on partition of protein and the alkaline protease (Table 1). The protein content in the crude extract was 4.8±0.58 mg/ml. It is seen that, for all the salts, the protein content (mg/ml) was higher in the bottom phase (0.35–0.72 mg/ml) and so was the protein yield (13.5–33.5%). Where as, in the top phase the protein yield was in the range of 8.6 to 21.6%, highest being in the ATPS of PEG and sodium sulphate. The protein partition coefficient ranged from 0.29 to 0.89 indicating that the partition of chicken intestine protein in the ATPS is affected by the salt used in the system. It was observed that the total protein yield (total of top and bottom phase) was in the range of 22.0 ($MgSO_4$) to 50.9% ($NaSO_4$), indicating the loss of protein due to precipitation, which formed the interface.

The specific activity of the crude enzyme extract (mg tyrosine released/mg protein/min) was 11.4±1.28 units. Upon ATPS separation the enzyme activity was higher in the top phase for all the salts (9.9–84.2). Higher enzyme activity (84.2) was found for top phase of PEG and sodium citrate followed by PEG and magnesium sulphate (69.1) and potassium phosphate (60.7) system. Sodium carbonate showed the least activity in both top and bottom phase indicating that the enzyme activity was lost in this system. The enzyme recovery in the top phase ranged from 11.0–73.5, the highest being in ATPS of PEG-potassium phosphate while in the bottom phase, it ranged from 7.9–78.5 with ATPS system of PEG-sodium citrate exhibiting the highest. The partition coefficient was in the range of 1.6–3.8. The enzyme purification fold in the top and bottom phase was also varying due to different ATPS system. The higher amount of purification was achieved in the top phase (up to 7.4 fold) as compared to that in the bottom phase (up to 3.8 fold).

Table 1 Protein content (P), protein yield (Yp), protein partition coefficient (Kp), enzyme activity (E-SA), enzyme yield (Ye), enzyme partition coefficient (Ke) purification factor (PF) and volume ratio (VR) in ATPS formed between PEG 6000 and different salts and crude enzyme extract ($n=3$) in ATPS formed between PEG 6000 and different salts and crude enzyme extract

Salt	Phase	P(mg/ml)	Yp	Kp	E-SA	Ye	Ke	PF	VR
Ammonium sulphate	Top	0.24 ± 0.02^{a}	15.1 ± 0.92^{a}	0.89 ± 0.04^{a}	30.3 ± 1.97^{a}	40.6 ± 5.91^{a}	1.7 ± 0.10^{a}	2.7 ± 0.36^{a}	2.4 ± 0.28^{a}
	Bottom	0.72 ± 0.13^{p}	17.1 ± 1.81^{p}		18.3 ± 2.29^{p}	27.6 ± 4.52^{p}		1.6 ± 0.29^{p}	
Magnesium sulphate	Top	0.13 ± 0.02^{b}	8.6 ± 1.15^{b}	0.63 ± 0.02^{b}	69.1 ± 7.65^{b}	51.9 ± 5.33^{b}	1.6 ± 0.19^{a}	6.1 ± 0.16^{b}	2.2 ± 0.12^{a}
	Bottom	0.46 ± 0.04^{q}	13.5 ± 1.61^{p}		42.2 ± 2.14^{q}	50.5 ± 8.48^{q}		3.8 ± 0.46^{q}	
Potassium phosphate	Top	0.28 ± 0.05^{a}	13.9 ± 1.52^{a}	0.41 ± 0.02^{c}	60.7 ± 11.05^{b}	73.5 ± 4.45^{c}	3.8 ± 0.22^{b}	5.3 ± 0.83^{b}	0.90 ± 0.06^{bc}
	Bottom	0.65 ± 0.01^{p}	33.5 ± 2.84^{q}		15.8 ± 3.22^{pr}	46.1 ± 3.06^{q}		1.34 ± 0.19^{pr}	
Sodium phosphate	Top	0.25 ± 0.05^{a}	10.5 ± 1.76^{bc}	0.43 ± 0.05^{cd}	18.9 ± 5.31^{ad}	16.9 ± 1.84^{d}	1.6 ± 0.20^{a}	1.6 ± 0.40^{c}	0.66 ± 0.05^{b}
	Bottom	0.35 ± 0.03^{q}	24.7 ± 2.22^{r}		12.1 ± 3.99^{rs}	25.5 ± 4.06^{p}		1.0 ± 0.25^{rt}	
Sodium citrate	Top	0.21 ± 0.02^{ab}	9.4 ± 0.73^{b}	0.29 ± 0.02^{e}	84.2 ± 7.37^{c}	69.7 ± 1.61^{c}	3.1 ± 0.37^{c}	7.4 ± 0.43^{d}	0.79 ± 0.05^{b}
	Bottom	0.50 ± 0.09^{q}	32.6 ± 0.49^{qr}		27.6 ± 5.33^{t}	78.7 ± 5.02^{r}		2.4 ± 0.19^{s}	
Sodium sulphate	Top	0.43 ± 0.11^{c}	21.6 ± 2.23^{d}	0.73 ± 0.05^{f}	30.2 ± 0.55^{a}	57.4 ± 3.98^{b}	3.6 ± 0.22^{b}	2.7 ± 0.33^{a}	1.0 ± 0.02^{c}
	Bottom	0.68 ± 0.02^{p}	29.3 ± 2.32^{s}		8.5 ± 0.40^{su}	22.1 ± 2.06^{p}		0.75 ± 0.05^{t}	
Sodium carbonate	Top	0.24 ± 0.02^{a}	12.8 ± 1.65^{ac}	0.49 ± 0.02^{d}	9.9 ± 2.46^{d}	10.9 ± 0.93^{d}	2.9 ± 0.17^{c}	0.86 ± 0.14^{e}	1.6 ± 0.21^{d}
	Bottom	0.67 ± 0.09^{p}	26.4 ± 2.83^{rs}		3.4 ± 0.68^{u}	7.9 ± 0.24^{s}		0.30 ± 0.03^{u}	

The protein content in the crude enzyme extract was 4.8±0.58 mg/ml. The specific activity of the crude enzyme extract is 11.4±1.28. Values in columns with same superscripts (a, b, c, d, e, f for top phase and p, q, r, s, t for bottom phase) do not differ significantly ($p<0.05$) ($n=3$)

The type of salts used in ATPS is known to affect the separation of protein and enzymes. Salabat (2001) demonstrated the influence of different salts on the phase composition in aqueous two phase systems. Klomklao et al. (2005) studied the effect of different salts on the partition behavior of proteinases from tuna spleen in ATPS and observed that ATPS system consisting of PEG 1000 and magnesium sulphate provided the best condition for maximum partitioning of proteinases in PEG rich top phase. As the partition behavior depends on type of proteinases also, in the present study the combination of PEG 6000 with potassium phosphate or sodium citrate was found to be more suitable for purification of alkaline protease from chicken intestine by ATPS. Further pH of the system also plays an important role in ATPS (Antov 2004; Tubio et al. 2007). In the present study the pH of the system ranged from 4.2 (PEG-sodium phosphate) to 11.1 (PEG-sodium carbonate), while the pH in PEG-potassium phosphate and PEG-sodium citrate was 6.7 and 8.2. The loss of enzyme activity in PEG-sodium carbonate system may be attributed to high pH values.

Effect of salt concentration Studies on effect of different salts on purification of alkaline protease from chicken intestine showed that ATPS of PEG with potassium phosphate or sodium citrate gives better enzyme purification. Thus further studies were conducted using these two salts. Data of Table 2 reveal that the bottom phase was rich in protein content (0.70–1.03) in various salt concentrations and hence was the protein yield (30.6–42.0). However, the protein content in the PEG rich top phase was not affected ($p\geq0.05$) by the concentration of the salt in the system. In case of potassium phosphate the increase in salt concentration resulted in decreased protein yield in the top phase, mainly due to difference in the volume ratio. However, no such difference in the protein yield was observed in PEG-sodium citrate system. The partition coefficient for sodium citrate varied from 0.32–0.39 and 0.20–0.24 for potassium phosphate. The volume ratio was highest in 15% concentration of respective salts and indicated that with increase in salt concentration the volume of top layer reduced. However, the protein content in the bottom phase was lower with increasing concentration of salt.

The enzyme activity was found to be higher in the top phase (40.2–80.5) and was highest for 15% sodium citrate (80.5) and 25% potassium phosphate (53.2). In case of PEG-sodium citrate system, the enzyme activity in both the phase reduced with increase in salt concentration indicating the loss of enzyme activity with increasing concentration of sodium citrate in the system. The enzyme yield in the top phase was highest (59.8±4.5%) for PEG-15% sodium citrate, while the enzyme activity in PEG-potassium phosphate system at all salt concentration was lower than the sodium citrate system. However, the partition coeffi-

Table 2 Protein content (P), protein yield (Yp), protein partition coefficient (Kp), enzyme activity (E-SA), enzyme yield (Ye), enzyme partition coefficient (Ke) purification factor (PF) and volume ratio (VR) ATPS formed between PEG 6000 and different concentration of salts (Sodium citrate and Potassium phosphate) and crude enzyme extract

Salt	Phase	P, mg/ml	Yp	Kp	E-SA	Ye	Ke	PF	VR
Sodium citrate (15%)	Top	0.33 ± 0.09^{NS}	10.2 ± 2.01^{a}	0.32 ± 0.05^{a}	80.5 ± 7.64^{a}	59.8 ± 4.56^{a}	2.4 ± 1.03^{a}	6.7 ± 0.91^{a}	0.81 ± 0.04^{a}
	Bottom	0.83 ± 0.18^{p}	32.0 ± 3.15^{p}		43.0 ± 1.36^{p}	82.3 ± 19.61^{p}		3.6 ± 0.37^{p}	
Sodium citrate (20%)	Top	0.42 ± 0.08^{NS}	12.1 ± 1.46^{a}	0.39 ± 0.05^{a}	43.2 ± 10.28^{b}	38.8 ± 7.14^{b}	1.6 ± 0.29^{a}	3.6 ± 0.55^{b}	0.70 ± 0.03^{b}
	Bottom	0.76 ± 0.07^{p}	31.3 ± 0.33^{p}		26.3 ± 1.81^{q}	77.3 ± 13.13^{p}		2.2 ± 0.06^{q}	
Sodium citrate (25%)	Top	0.39 ± 0.05^{NS}	10.4 ± 0.64^{a}	0.34 ± 0.05^{a}	40.2 ± 14.16^{b}	34.7 ± 9.81^{b}	3.5 ± 0.69^{ab}	3.4 ± 1.13^{b}	0.61 ± 0.03^{c}
	Bottom	0.70 ± 0.02^{p}	30.6 ± 2.66^{p}		11.3 ± 2.40^{r}	31.3 ± 3.66^{q}		0.94 ± 0.16^{r}	
Potassium phosphate (15%)	Top	0.33 ± 0.02^{NS}	9.9 ± 0.91^{a}	0.24 ± 0.04^{ab}	49.6 ± 10.01^{b}	33.8 ± 13.91^{b}	3.4 ± 0.69^{ab}	4.1 ± 0.63^{b}	0.82 ± 0.03^{a}
	Bottom	1.0 ± 0.09^{q}	41.9 ± 3.92^{q}		14.4 ± 0.90^{r}	55.2 ± 4.34^{r}		1.2 ± 0.06^{r}	
Potassium phosphate (20%)	Top	0.33 ± 0.08^{NS}	8.6 ± 0.81^{ab}	0.21 ± 0.03^{b}	47.8 ± 20.74^{b}	27.1 ± 13.66^{b}	$5.1\pm2.23b^{c}$	3.9 ± 1.46^{b}	0.59 ± 0.07^{c}
	Bottom	0.94 ± 0.03^{pqr}	41.7 ± 2.83^{q}		9.5 ± 0.70^{r}	35.5 ± 1.82^{q}		0.79 ± 0.12^{r}	
Potassium phosphate (25%)	Top	0.27 ± 0.10^{NS}	6.5 ± 1.54^{b}	0.20 ± 0.07^{b}	53.2 ± 22.93^{b}	24.3 ± 10.74^{b}	6.5 ± 1.68^{c}	4.4 ± 1.67^{b}	0.54 ± 0.02^{c}
	Bottom	0.75 ± 0.03^{pr}	34.8 ± 4.37^{p}		8.3 ± 2.69^{r}	26.6 ± 8.03^{q}		0.68 ± 0.17^{r}	

The protein content in the crude enzyme extract is 5.7±0.55 mg/ml. The specific activity of the crude enzyme extract is 12.0±1.15.

Values in columns with same superscripts (a, b, c for top phase and p, q, r for bottom phase) do not differ significantly ($p<0.05$). NS-not significant ($p<0.05$). ($n=3$)

Table 3 Protein content (P), protein yield (Yp), protein partition coefficient (Kp), enzyme activity (E-SA), enzyme yield (Ye), enzyme partition coefficient (Ke) purification factor (PF) and volume ratio (VR) in ATPS formed between PEG 6000-Sodium citrate and different levels of crude enzyme extract (CE)

Protein concentration, mg/ml	Phase	P, mg/ml	Yp	Kp	E-SA	Ye	Ke	PF	VR
1.75	Top	0.33 ± 0.05^{a}	8.6 ± 1.04^{a}	0.26 ± 0.06^{a}	36.2 ± 5.68^{ab}	37.3 ± 3.71^{a}	2.4 ± 0.33^{NS}	4.8 ± 0.66^{ab}	0.86 ± 0.04^{NS}
	Bottom	1.3 ± 0.15^{p}	39.3 ± 3.55^{p}		15.3 ± 0.37^{p}	86.3 ± 5.41^{NS}		2.0 ± 0.12^{p}	
1.5	Top	0.30 ± 0.04^{ab}	9.1 ± 1.16^{ab}	0.27 ± 0.06^{a}	42.1 ± 6.54^{a}	45.3 ± 1.42^{bc}	3.1 ± 0.60^{NS}	5.6 ± 0.61^{a}	0.85 ± 0.03^{NS}
	Bottom	1.1 ± 0.08^{qr}	39.9 ± 2.61^{p}		13.7 ± 0.97^{pq}	79.4 ± 1.43^{NS}		1.8 ± 0.17^{pq}	
1.25	Top	0.26 ± 0.04^{abc}	9.7 ± 1.42^{ab}	0.27 ± 0.07^{a}	35.7 ± 2.33^{ab}	41.6 ± 3.01^{abc}	2.6 ± 0.39^{NS}	4.7 ± 0.32^{ab}	0.86 ± 0.04^{NS}
	Bottom	0.98 ± 0.08^{qr}	42.0 ± 3.12^{pq}		13.4 ± 1.18^{pq}	82.5 ± 1.66^{NS}		1.8 ± 0.20^{pq}	
1.0	Top	0.25 ± 0.04^{bc}	11.5 ± 1.76^{b}	0.31 ± 0.07^{a}	34.1 ± 5.73^{ab}	46.5 ± 4.07^{bc}	2.4 ± 0.48^{NS}	4.6 ± 0.74^{ab}	0.88 ± 0.02^{NS}
	Bottom	0.82 ± 0.08^{r}	43.4 ± 3.56^{pq}		14.6 ± 1.23^{p}	94.0 ± 15.72^{NS}		1.9 ± 0.10^{p}	
0.5	Top	0.22 ± 0.01^{c}	20.2 ± 1.23^{c}	0.46 ± 0.07^{b}	29.8 ± 3.08^{b}	74.0 ± 2.28^{d}	2.5 ± 0.21^{NS}	4.0 ± 0.25^{b}	0.90 ± 0.06^{NS}
	Bottom	0.49 ± 0.06^{s}	49.9 ± 7.74^{q}		11.9 ± 0.75^{q}	90.9 ± 15.74^{NS}		1.6 ± 0.12^{q}	

The protein content in the crude enzyme extract is 5.7±1.12. The specific activity of the crude enzyme extracts is 7.5±0.37.

Values in columns with same superscripts (a, b, c, d for top phase and p, q, r, s for bottom phase) do not differ significantly ($p<0.05$). NS-not significant ($p<0.05$). ($n=3$)

cient varied in the range of 1.6–3.5 and 3.4–6.5 for different concentrations of sodium citrate and potassium phosphate respectively. The purification fold was highest in the top phase for both the salts. For 15% sodium citrate, purification of 6.7 fold was achieved in its top phase. However, in case of PEG-Potassium phosphate system, highest purification (4.4 fold) was achieved with 25% potassium phosphate in the system but was not significantly ($p \geq 0.05$) affected by the salt concentration.

Partition of proteins and enzymes in ATPS was found to be affected by the type and concentration of salt in the system (Yue et al. 2007; Ravindra Babu et al. 2008). Different studies used different salts/PEG at different concentration for different enzymes. As the partition of biomolecules depends on various factors and influenced by experimental condition (Rito-Palomares 2004), it is difficult to compare the results of present study with the reported findings.

Effect of protein concentration As, PEG-sodium citrate system gave higher enzyme yield and enzyme purification further studies were done with this system. The protein content in the crude enzyme extract was 5.7±1.12. Protein recovery in both the phases increased with decrease in protein concentration and the highest protein recovery was seen in the protein concentration of 0.5 mg/ml (Table 3). As the protein concentration is increased in the system, higher quantity of proteins is precipitated resulting in the decreased protein yield. The partition coefficient increased with decrease in protein concentration indicating the better separation of protein into the top layer with reduced protein concentration of the sample. However, the protein concentration in the system did not affect ($p \geq 0.05$) volume ratio (0.85–0.90), but higher volume of upper phase was observed with decreasing protein concentration.

The enzyme activity in crude extract was 7.5±0.37. Upon ATPS separation enzyme activity was found higher in the top phase (29.8–42.1) than bottom phase (11.9–15.3). However, in general enzyme activity reduced and enzyme yield increased in top phase with decrease in protein concentration. The enzyme recovery was higher in bottom phase (79.4–94.0) but did not differ with change in protein concentration. The enzyme purification factor did not show any specific trend and was in the range of 4.0–5.6 fold and 1.6–2.0 fold in top and bottom phase respectively. Nitsawang et al. (2006) reported that change in protein concentration do not have any notable effect of partition of papain in ATPS, but the enzyme yield decreases with increased protein concentration. Similar trend was also observed in the present study.

Effect of temperature Enzymes are highly heat sensitive biomolecules. Thus it is important to evaluate the effect of temperature on the partition behavior and activity of enzymes in ATPS. Except for protein content in bottom phase none of the other parameters were found to be significantly affected by the temperature ($p \leq 0.05$) (Table 4). ATPS systems kept at different temperatures of 20°C, room temperature (RT), 37 and 45°C showed higher protein content and yield in their respective bottom phases. ATPS system at 20°C had the highest protein content (1.2 mg/ml and 0.39 mg/ml in bottom and top phase respectively) and highest yield (total of 62.7%). The partition coefficient varied from 0.32–0.37 and volume ratio from 0.79–0.88, but no significant ($p > 0.05$) differences were observed due to temperature.

The activity of crude enzyme was 9.9±2.08. Enzyme activity was found to be higher in the top phases in different temperatures with 20°C having the highest value (37.8 and 15.9 in upper and lower phase, respectively) but was not significantly different from ATPS at other temperatures. In general with increase in temperature the enzyme activity reduced and correspondingly the enzyme yield also. The

Table 4 Protein content (P), protein yield (Yp), protein partition coefficient (Kp), enzyme activity (E-SA), enzyme yield (Ye), enzyme partition coefficient (Ke) purification factor (PF) and volume ratio (VR) in ATPS formed between PEG 6000 and sodium citrate at different temperatures

Temp., °C	Phase	P, mg/ml	Yp	Kp	E-SA	Ye	Ke	PF	VR
20	Top	0.39±0.01	13.6±2.11	0.32±0.01	37.8±9.46	56.8±13.91	2.5±0.45	3.8±0.44	0.88±0.05
	Bottom	1.23 ± 0.06^{p}	49.0±6.43		15.9±6.32	78.9±22.02		1.6±0.40	
27±2 (RT)	Top	0.39±0.04	13.5±4.12	0.35±0.04	33.2±11.40	47.2±13.63	2.3±0.62	3.3±0.63	0.83±0.05
	Bottom	1.1 ± 0.06^{pq}	46.2±6.04		14.7±3.42	71.8±18.64		1.5±0.41	
37	Top	0.36±0.02	11.9±2.28	0.37±0.04	34.0±10.98	46.0±16.71	2.4±0.74	3.4±0.38	0.81±0.02
	Bottom	0.97 ± 0.13^{qr}	39.9±8.31		14.4±2.18	62.6±15.78		1.5±0.43	
45	Top	0.34±0.05	11.4±3.27	0.37±0.06	30.1±5.88	37.6±9.05	2.6±0.59	3.0±0.21	0.79±0.09
	Bottom	0.93 ± 0.13^{r}	38.9±8.49		12.2±2.91	52.2±18.04		1.3±0.42	

RT: Room temperature; The protein content in the crude enzyme extract is 5.4±0.77. The specific activity of the crude enzyme extracts is 9.9±2.08.

Values in columns with same superscripts (p, q, r for protein content in bottom phase) do not differ significantly ($p<0.05$). All the other parameters were not significant ($p<0.05$). ($n=3$)

Table 5 Protein content (P), protein yield (Yp), protein partition coefficient (Kp), enzyme activity (E-SA), enzyme yield (Ye), enzyme partition coefficient (Ke) purification factor (PF) and volume ratio (VR) in ATPS formed between different concentrations of PEG 6000 and 15% sodium citrate

PEG 6000 (%)	Phase	P, mg/ml	Yp	Kp	E-SA	Ye	Ke	PF	VR
15	Top	0.29 ± 0.03^{a}	10.2 ± 0.23^{a}	0.42 ± 0.02^{a}	57.1 ± 3.10^{a}	53.7 ± 4.05^{a}	1.5 ± 0.10^{a}	5.0 ± 0.30^{a}	0.73 ± 0.02^{a}
	Bottom	0.68 ± 0.06^{p}	33.4 ± 1.34^{p}		38.2 ± 0.75^{p}	112.1 ± 9.92^{p}		3.4 ± 0.21^{p}	
20	Top	0.34 ± 0.02^{ab}	13.7 ± 0.81^{b}	0.31 ± 0.01^{b}	26.3 ± 2.73^{b}	30.8 ± 2.33^{b}	1.2 ± 0.15^{b}	2.3 ± 0.10^{b}	0.91 ± 0.02^{b}
	Bottom	1.1 ± 0.06^{q}	48.8 ± 2.45^{q}		21.4 ± 0.67^{q}	93.3 ± 12.84^{p}		1.9 ± 0.16^{q}	
25	Top	0.36 ± 0.02^{b}	16.1 ± 0.86^{c}	0.29 ± 0.01^{b}	15.3 ± 0.94^{c}	21.9 ± 1.57^{c}	1.2 ± 0.07^{b}	1.4 ± 0.01^{c}	1.2 ± 0.03^{c}
	Bottom	1.2 ± 0.04^{r}	48.5 ± 3.24^{q}		12.7 ± 0.69^{r}	54.7 ± 6.34^{q}		1.1 ± 0.06^{r}	

The protein content in the crude enzyme extract was 4.8±0.54 mg/ml. The specific activity of the crude enzyme extracts was 11.4±0.76.

Values in columns with same superscripts (a, b, c for top phase and p, q, r for bottom phase) do not differ significantly ($p<0.05$). ($n=3$)

enzyme partition coefficient did not show any marked changes with changes in incubation temperature. The purification fold was also highest at 20°C of 3.8 fold and 1.6 fold in top and bottom phase respectively as compared to other higher temperature. The decrease in enzyme activity, enzyme yield and enzyme purification, even though was marginal, can be attributed the detrimental effect of higher temperature on proteases. Reports are available on the effect of temperature on the partition behavior of enzymes in aqueous-two phase systems (Tubio et al. 2007).

Effect of PEG concentration The protein content in the crude extract was 4.8±0.54 mg/ml. The protein content was higher in the bottom phase (0.68–1.24 mg/ml) than the upper phase (0.29–0.36 mg/ml) (Table 5). With increase in PEG concentration, the protein content also increased in both the phases. The same trend was observed in the amount of protein recovery, which ranged from 33.4–48.8 and 10.2–16.1% in the bottom and top phase respectively. Volume ratio increased with an increase in PEG concentration from 0.73 to 1.2 and correspondingly the partition coefficient decreased from 0.42 to 0.29 with increase in PEG concentration.

The specific activity of the crude enzyme was 11.4±0.76 units. The activity of alkaline proteases decreased with increase in the PEG concentration. The enzyme yield, partition coefficient and purification fold was highest at low concentration of PEG and their value reduced with an increase in PEG concentration. Similarly the enzyme purification was higher at lower concentration of PEG. Polymer concentration and molecular weight is known to affect the partition behaviour of biomolecules in ATPS (Diamond and Hsu 1992). The reports on effect of PEG concentration on partitioning of enzymes revealed that, it is dependent on the type of enzyme. Nitsawang et al. (2006) did not observe any difference in the partition of papain in ATPS formed between different concentration of PEG 6000 and ammonium sulphate. However, Sinha et al. (1996) observed the significant effect of PEG concentration on the partition of bacterial alkaline protease.

Effect of pH The pH is an important factor which determines the partition behaviors of biomolecules in ATPS. The effect

Table 6 Protein content (P), protein yield (Yp), protein partition coefficient (Kp), enzyme activity (E-SA), enzyme yield (Ye), enzyme partition coefficient (Ke) purification factor (PF) and volume ratio (VR) in ATPS formed between 20% PEG 6000 and 15% sodium citrate at different pH

pH	Phase	P, mg/ml	Yp	Kp	E-SA	Ye	Ke	PF	VR
7	Top	0.27 ± 0.05^{NS}	12.0 ± 1.14^{a}	0.25 ± 0.04^{a}	30.8 ± 4.79^{NS}	36.1 ± 4.61^{a}	1.6 ± 0.21^{a}	3.0 ± 0.44^{NS}	1.1 ± 0.12^{a}
	Bottom	1.1 ± 0.04^{p}	45.3 ± 2.06^{p}		19.0 ± 1.24^{p}	83.9 ± 1.24^{p}		1.8 ± 0.06^{p}	
8	Top	0.29 ± 0.03^{NS}	12.1 ± 0.73^{a}	0.26 ± 0.02^{a}	31.5 ± 2.04^{NS}	37.5 ± 4.73^{a}	1.8 ± 0.23^{ac}	3.1 ± 0.27^{NS}	0.96 ± 0.04^{a}
	Bottom	1.1 ± 0.03^{p}	48.8 ± 3.44^{p}		17.6 ± 1.11^{p}	84.0 ± 5.92^{p}		1.7 ± 0.24^{p}	
9	Top	0.26 ± 0.03^{NS}	13.0 ± 0.65^{a}	0.52 ± 0.05^{b}	34.8 ± 4.85^{NS}	44.2 ± 3.27^{ab}	0.83 ± 0.04^{b}	3.4 ± 0.39^{NS}	1.4 ± 0.14^{b}
	Bottom	0.51 ± 0.02^{q}	18.4 ± 0.81^{q}		41.8 ± 3.73^{q}	75.7 ± 9.34^{p}		4.1 ± 0.35^{q}	
10	Top	0.32 ± 0.02^{NS}	15.5 ± 1.52^{b}	0.79 ± 0.01^{c}	30.5 ± 3.89^{NS}	46.2 ± 3.95^{b}	2.0 ± 0.11^{c}	3.0 ± 0.12^{NS}	1.3 ± 0.05^{b}
	Bottom	0.40 ± 0.02^{r}	14.7 ± 1.18^{q}		15.1 ± 2.05^{p}	21.6 ± 0.61^{q}		1.5 ± 0.12^{p}	

The protein content in the crude enzyme extract was 4.65±0.30. The specific activity of the crude enzyme extracts is 10.27±0.98.

Values in columns with same superscripts (a, b, c for top phase and p, q for bottom phase) do not differ significantly ($p<0.05$). NS-not significant ($p<0.05$). ($n=3$)

of pH on separation of chicken intestine protein and alkaline protease in ATPS of PEG-sodium citrate is presented in Table 6. As observed earlier, the protein content was higher in the bottom phase (0.32–1.1 mg/ml) than that in the top phases (0.26–0.32 mg/ml). The protein recovery was also higher in bottom phase than the top phase. Protein recovery was highest at pH 8.0 (total 60.9%). With further increase in pH protein recovery reduced.

Enzyme activity in the top phase was not affected ($p \geq 0.05$) by the change in the pH of the system, with highest (34.8 units) at pH 9. Yield of enzyme in top phase was found to increase with increase pH with a corresponding decrease in enzyme yield in the bottom phase. Even though, the purification fold of enzyme in top phase was not affected by the pH, it was higher at pH 9. It is important to note that although the enzyme yield was highest at pH 10, the purification fold was lower. The pH was found to play an important role in separation of enzymes in ATPS due to inactivation of proteases below or above a specific pH (Schmidt et al. 1994; Nitsawang et al. 2006).

Conclusion

The study indicated the influence of various factors on separation and purification of alkaline proteases from chicken intestine in aqueous two phase systems. ATPS between PEG 6000 and sodium citrate was found to give higher enzyme purification compared to other salts. Factors such as salt concentration, initial protein concentration, temperature, pH and concentration of PEG had variable effect on partition of alkaline proteases from chicken intestine in aqueous two phase system. It can be concluded that for efficient separation of alkaline proteases from chicken intestine, an ATPS of 15% PEG and 15% sodium citrate, initial protein concentration of 1.0–1.5 mg/ml in the system, a temperature of 20°C, with a pH of 8–9 was more suitable. The study indicated that ATPS can be used as a preliminary step in purification of alkaline proteases from chicken intestine.

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