Efficient Clarification of the Hepatopancreatic Homogenate of Red Shrimp During the Recovery of Crude Alkaline Phosphatase

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ABSTRACT

Centrifugal force and time for the primary clarification of hepatopancreatic homogenate of red shrimp was determined on the optimum recovery of alkaline phospatase. Centrifugal force of 1681.1×g for 5 min or more than 1681.1×g for 5, 10, 15, or 20 min successfully clarify 96.99±0.45% of the total solids, 93.16±1.03% of soluble protein, and 93.61±0.16% of the lipids from the respective initial homogenate. However, the centrifugal force more than 1681.1×g caused denaturation of alkaline phosphatase due to hydrodynamic sheer even at the temperature of 4^{o}C. Centrifugal force of 1681.1×g for 5 min was able to retain 94.29±1.44% of the alkaline phosphatase of the initial homogenate, eventhough clarified 89.98±0.01% of the total solids, 77.25±0.89% of soluble protein, and 78.76±0.64% of the lipids from the respective initial homogenate. Centrifugal force of 1681.1×g for 5 min is an efficiently removes insoluble while retaining optimum alkaline phosphatase activity.

Key words: Alkaline phosphatase, Red shrimp, Insoluble, Centrifugation, Clarification, Hepatopancreas.

INTRODUCTION

Marine environment contains a large pool of diversified species adapted to a variety of habitat conditions is an important source of enzymes with unique novel properties that can be commercially exploited in medicine, laboratory, dairy, leather processing and other bioprocess industries. Red shrimp (*Penaeus indicus*) is caught regularly all along coastal Karnataka during fishing season to produce value added seafood products for international market leaving behind large amount of hepatopancreatic wastes that is a very good source of alkaline phosphatase. Alkaline phosphatase (EC 3.1.3.1) is non-specific phosphomonoesterase that are ubiquitous in nature, diverse in some of their physic-chemical properties due to adaptation, and associated with different types of impurities. Careful strategy has to be adopted to remove insoluble during the initial stage of enzyme purification itself to prevent the interference of these components on the subsequent purification (Chen *et al*., 2012). Centrifugation is an effective method

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compared to other process alternatives to clarify the hepatopancreatic homogenates in which alkaline phosphatase is already released into the medium (Iammarino *et al*., 2007). Removal of undesirable insoluble such as cell debris, organelles and lipids of the tissue homogenates is a significant bottle-neck in primary clarification because these components shows variation in quantity, ratio and physic-chemical properties (Shringari *et al*., 2012). Careful selection of clarification parameters such as relative centrifugal force and resident time in centrifuge ensures consistent and reproducible enzyme yield (Yavorsky *et al*., 2003). Low relative centrifugal force and centrifugation time partially clarifies the tissue homogenate, and high relative centrifugal force and centrifugation time may denature the enzyme due to hydrodynamic sheer force of centrifugation even though improves the clarification (Takagi *et al*., 2000; Elias and Joshi, 1998). Efficient clarification parameter affects the purification strategy and cost effectiveness of the process. Carefully designed enzyme recovery process adopting a efficient primary clarification strategy based on laboratory test values improves the efficiency of the purification process (Roush and Lu, 2008). Numbers of efforts were made by various researchers to remove insoluble and extract protein by centrifugation from biological tissue homogenates (Maybury *et al*., 2000, Hutchison, 2006, Wang *et al*., 2006). So far no single set of standard operating conditions such as centrifugal force and resident time have been defined to isolate enzymes from the tissue homogenates to achieve optimum activity, yield and purity. Objective of the present work is to investigate the efficiency of relative centrifugal forces and resident time in the centrifuge on the primary clarification of the hepatopancreatic tissues of Red shrimp so as to set a standard operating condition that could be used for isolating alkaline phosphatase from the with optimum yield, purity and activity.

MATERIALS AND METHODS

Chemicals

All the chemicals and reagents used were of analytical grade and were obtained from Merck Limited (Mumbai, India). Solutions were prepared using reagents according to the current American Chemical Society specifications (1999). Buffer used for the homogenisation of hepatopancreatic tissues of shrimps was 0.1 *M* Tris-HCl buffer of pH 8.4, and buffer used for the enzyme assay was 2-amino-2-methyl-1-propanol (AMP) buffer of pH 10.3. Buffer preparations were filtered and sterilized in moist heat at 121^{o}C for 20 min.

Sample collection

Freshly caught Red shrimps (*Solenocera choprai*) from the Arabian Sea using trawl nets belonging to size group of 34-66 mm in length, and weighing around 15-25 g were transported to the laboratory after adequately icing them in the proportion of 1:1 shrimp to ice, washed using chilled running water system, identified as per Racek (1955), and dissected to remove the hepatopancreas. The hepatopancreas and attached tissues were sorted, weighed, packed, labeled, frozen at –40^{o}C, and stored at –20^{o}C in a deep freezer (JHBio, Chennai, India) until further use.

Homogenization

Hepatopancreas and attached tissues were thawed at room temperature of about 28°C, weighed and homogenized using a Potter-Elvehjem homogenizer (RH-2 Homogenizer, Rotek Instruments, Kerala, India) with a sample holding tank mounted in a cooling jacket maintained at 4°C. Homogenisation was carried out at pestle head speed of 3,000 rpm for 10 min using 0.1 *M* Tris-HCl buffer at 1:10 tissue to buffer ratio.

Centrifugation

Clarification experiment was carried out in microprocessor controlled low volume (1 mL) high speed refrigerated centrifuge of model C-24BL/CRP24 (Remi Laboratory Instruments, Mumbai, India) fitted with R-248 model 24x1.5 mL fixed-angle rotor. The crude homogenate was centrifuged at relative centrifugal force (RCF) of 67.2, 1681.1, 6724.3, 15124.8 or 26897.4×g for 5, 10, 15, or 20 min at 4°C. Separated infranatant, and reconstituted pellets and supernatant in 0.1 *M* Tris-HCl was estimated for total solid content, protein content, fat content and alkaline phosphatase activity.

Proximate analysis

Samples were drawn at different intervals and force of centrifugation was performed in quadruplicates. Moisture content and solid content of the samples were estimated as per the guidelines of Food and Agriculture Organization of the United Nations (1994), and expressed as percentage moisture. The protein content was estimated as per the Folin-Ciocalteu method of Lowry *et al*. (1951) and expressed as mg/L, using bovine serum albumin (BSA) as a standard. The lipids were quantitatively estimated by Sulpho-phosphovanillin method of Barnes and Blackstock and expressed as mg/L (Barnes and Blackstock, 1973).

Enzyme assay

Alkaline phosphatase assay was performed using an artificial substrate as per the method explained by Bomers and McComb using disodium paranitrophenyl phosphate (*p*NPP) as an artificial substrate and disodium paranitrophenol (*p*NP) as a standard (1975). Alkaline phosphatase activity in units/L is the liberation of 1 m*M* of *p*NP per min at 37°C incubation temperature per liter of tissue homogenate in respective buffers.

Statistical analysis

The analysis of cell disruption was carried out in quadruplicate. The results were treated by analysis of variance (ANOVA), followed by Tukey's test, using the software Statistica 6.0 (Statsoft, Tulsa, OK, USA). The results were expressed as averages±standard deviations followed by corresponding letters which indicates the significant differences. All analyses were performed considering a confidence level of 95% ($p<0.05$).

RESULTS

Effectiveness at which insoluble are removed by various centrifugal force and centrifugation time from the Red shrimp

homogenate was done by estimating total solids, protein, lipid and alkaline phosphatase activity in supernatant, infranatant, and pellet.

Efficiency of different centrifugal force and time on fractionation of solids

Total solids in the hepatopancreatic tissue homogenates of Red shrimp were estimated as 20.00±0.56%. It is interesting to note here that even though RCF of 67.2×g was able to clarify 63.50±0.22% of the total solids by 20 min of centrifugation, 56.21± 0.12% of the total solids of the homogenate were clarified before 5 min of centrifugation at this force. Whereas, RCF of 1681.1×g was able to clarify 89.98±0.01% of the total solids by 5 min of centrifugation, and 95.91 ±0.36% of the total solids by 10 min of centrifugation from the tissue homogenates. In our study were not able to establish any significant (p>0.05) difference in the solid content amongst the homogenates produced at 10, 15 and 20 min of centrifugation at 1681.1×g, or at 5, 10, 15 and 20 min of centrifugation at RCF of 6724.3, 15124.8, or 26897.4×g (Table. 1). Resident period of more than 5 min in the centrifuge at RCF of 1681.1×g or RCF of more than 1681.1×g for 5, 10, 15, or 20 min at 4^{o}C was able clarify 96.99±0.45% of the total solids, retaining only 3.01±0.45% of the solids of the respective tissue homogenate.

Table 1. Fractionation of solids of the homogenate at different centrifugal force and time

Relative Centrifugal Force (×g)	Resident time (min)	Solids (%)		
		Supernatant	Infranatant	Pellets
67.2	5	0.27±0.02	8.76±0.09	10.98±0.06
	10	0.68±0.03	7.72±0.06	11.61±0.08
	15	0.82±0.04	7.35±0.07	11.85±0.07
	20	0.84±0.09	7.30±0.05	11.88±0.58
1681.1	5	2.93±0.87	2.00±0.07	15.14±0.27
	10	3.40±0.75	0.82±0.03	15.78±0.33
	15	3.40±0.23	0.80±0.33	15.79±0.33
	20	3.43±0.42	0.73±0.12	15.93±0.23
6724.3	5	3.41±0.44	1.59±0.46	15.81±0.55
	10	3.44±0.23	0.72±0.65	15.84±0.66
	15	3.45±0.44	0.68±0.56	15.87±0.56
	20	3.48±0.36	0.61±0.67	16.00±0.66
15124.8	5	3.42±0.53	0.76±0.80	15.82±0.57
	10	3.44±0.26	0.70±0.88	15.85±0.78
	15	3.45±0.44	0.68±0.86	15.87±0.56
	20	3.51±0.36	0.54±0.78	16.05±0.33
26897.4±0.56	5	3.44±0.44	0.71±0.74	15.85±0.55
	10	3.48±0.23	0.62±0.46	15.90±0.46
	15	3.49±0.33	0.59±0.07	15.92±0.53
	20	3.51±0.23	0.54±0.09	16.05±0.34

Protein fractionation at different centrifugal force and time

Of the total estimated protein content of 5333.00±84.84 mg/L in the homogenate underwent fractionation at different centrifugal force and centrifugation time. Centrifugation of the homogenate even up to 20 min at RCF of 67.2×g was able to fractionate only 17.12±0.39% of the total soluble protein of the homogenate. At RCF of 1681.1×g, 5 min of the centrifugation fractionate only 77.25±0.89% of the protein, but 10 min of centrifugation was able to fractionate 90.76±0.99% of the protein into pellets. Here, no significant (p>0.05) difference in the protein content was registered amongst the pellets collected at the end of 10, 15, and 20 min of centrifugation at RCF of 1681.1×g, or at RCF of 6724.3, 15124.8, or 26897.4×g, for 5, 10, 15, or 20 min, as estimated by One-way ANOVA with *post hoc* Tukey's test (Table 2). All along these period at any centrifugal force no protein accumulation (p>0.05) was registered in the supernatant.

Table 2. Fractionation of proteins of the homogenate at different centrifugal force and time.

Relative Centrifugal Force (×g)	Resident time (min)	Protein content mg/L		
		Supernatant	Infranatant	Pellets
67.2	5	0	5303.25±55.34	29.25±01.26
	10	0	4671.25±53.17	661.25±12.23
	15	0	4449.75±32.88	882.75±06.56
	20	0	4419.75±52.94	912.75±04.56
1681.1	5	0	1213.00±63.89	4119.50±03.56
	10	0	495.00±67.12	4837.50±52.56
	15	0	486.50±61.75	4846.00±32.89
	20	0	440.00±55.44	4892.50±66.56
6724.3	5	0	470.75±49.51	4861.32±78.65
	10	0	438.25±40.73	4894.25±65.54
	15	0	414.00±09.76	4918.50±45.65
	20	0	371.00±40.12	4961.50±25.46
15124.8	5	0	457.25±09.43	4875.25±63.45
	10	0	426.50±74.88	4906.00±54.65
	15	0	409.50±50.82	4923.00±25.98
	20	0	324.00±05.89	5008.50±56.23
26897.4	5	0	432.25±57.78	4900.25±65.32
	10	0	376.50±18.43	4956.00±23.56
	15	0	359.50±07.05	4973.00±46.89
	20	0	324.00±81.05	5008.50±55.34

Lipid Fractionation at different centrifugal force and time

Total lipid content of the homogenate was estimated as 3721±22.4 mg/L. Even at the end of 20 min of centrifugation, RCF of 67.2×g was able fractionate only 22.60±0.09% of the lipids of the homogenates underwent floatation to form a fully developed layer on top of the medium. However at RCF of 1681.1×g, 78.76±0.64% of lipid lost from the homogenate at the end of 5 min of centrifugation, and 91.33±0.23% of the lipid lost from the homogenate at the end of 10 min of centrifugation. Nevertheless, further increase in the centrifugation period even up to 20 min at RCF of 1681.1×g, or increasing the RCF of beyond 1681.1×g up to 20 min was not able to change (p>0.05) any lipid content in the homogenate or the supernatant (Table. 3).

Table 3. Fractionation of lipids of the homogenate at different centrifugal force and time.

Relative Centrifugal Force (×g)	Resident time (min)	Lipid content mg/L		
		Supernatant	Infranatant	Pellets
67.2	5	265.31±05.23	3455.69±45.65	0
	10	677.13±06.45	3043.87±54.65	0
	15	821.47±09.56	2899.53±23.25	0
	20	841.01±04.45	2879.99±23.25	0
1681.1	5	2330.59±26.54	790.41±12.32	0
	10	3398.45±16.78	322.55±06.54	0
	15	3403.99±32.88	317.01±06.45	0
	20	3434.29±26.45	286.71±05.56	0
6724.3	5	3414.25±65.54	306.75±04.32	0
	10	3435.43±14.15	285.57±13.23	0
	15	3451.23±54.45	269.77±18.32	0
	20	3479.25±24.65	241.75±23.21	0
15124.8	5	3423.05±54.24	297.95±13.23	0
	10	3443.09±65.54	277.91±15.65	0
	15	3454.16±45.32	266.84±13.23	0
	20	3509.88±33.66	211.12±22.56	0
26897.4	5	3439.34±45.32	281.66±13.25	0
	10	3475.67±13.23	245.33±11.11	0
	15	3486.74±45.65	234.26±07.09	0
	20	3509.88±69.96	211.12±09.09	0

Fractionation of the enzyme activity at different centrifugal force and time

The homogenate lost only 7.63±1.47 % enzyme activity during entire 20 min of centrifugation at RCF of 67.2×g (Table 4). RCF of 1681.1×g was able to retain 94.29± 1.44% of the activity of the homogenate end of 5 min of centrifugation. However, when the homogenization period was increased to 10 min at RCF of to 1681.1×g, homogenate lost 51.13±1.33% of the activity even when the temperature was maintained at 4^{o}C. In this study, centrifugation of the homogenates at and beyond 10 min at RCF of to 1681.1×g or beyond RCF of to 1681.1×g even at 5 min resulted in significant (p<0.05) loss in the activity of the alkaline phosphatase.

Table 4. Fractionation of proteins of alkaline phosphatase activity in the homogenate at different centrifugal force and time.

Relative Centrifugal Force (×g)	Resident time (min)	Alkaline phosphatase activity units/L		
		Supernatant	Infranatant	Pellets
67.2	5	0	156.25±04.35	5.00±00.09
	10	0	154.50±04.65	13.00±00.11
	15	0	153.00±01.41	15.25±00.06
	20	0	150.50±07.05	16.38±00.06
1681.1	5	0	156.75±03.59	6.25±00.11
	10	0	85.00±04.56	15.13±00.09
	15	0	83.88±10.51	14.88±00.12
	20	0	83.25±08.46	10.38±00.06
6724.3	5	0	86.25±03.59	5.75±00.16
	10	0	79.38±06.54	15.25±00.06
	15	0	75.25±10.52	11.75±00.11
	20	0	71.63±09.18	13.88±00.06
15124.8	5	0	82.13±01.38	12.25±00.09
	10	0	78.13±04.50	14.63±00.11
	15	0	74.00±03.58	13.88±00.12
	20	0	71.63±03.58	15.50±00.09
26897.4	5	0	87.13±07.09	11.25±00.06
	10	0	75.63±05.72	10.23±00.09
	15	0	69.00±07.49	10.63±00.11
	20	0	61.63±07.92	16.50±00.02

Specific activity of homogenate at RCF of 67.2×g even upto 20 min in the centrifuge remained at a level of 0.031± 0.001 units/mg (Table 5). Here, even after registering same level of alkaline phosphatase activity in all the infranatants produced either at 67.2×g for 5, 10, 15, or 20 min, or at RCF of 1681.1×g for 5 min, specific activity of the infranatant centrifuged at RCF of 1681.1×g for 5 min increased by more than two folds of its initial homogenate. Beyond 5 min of centrifugation at RCF of 1681.1×g or beyond RCF of 1681.1×g for 5, 10, 15 or 20 min the specific activity of the tissue homogenates increased significantly (p<0.05) compared to homogenates obtained at RCF of 1681.1×g for 5 min, but centrifugation of the homogenates at and beyond 10 min resulted in significant (p<0.05) loss in the activity of the alkaline phosphatase.

Table 5. Changes in the specific activity of the homogenate at different centrifugal force and time.

Relative Centrifugal Force (×g)	Resident time (min)	Specific activity units/mg		
		Supernatant	Infranatant	Pellets
67.2	5	0	0.029±0.001	0.171±0.001
	10	0	0.030±0.001	0.019±0.001
	15	0	0.031±0.001	0.017±0.001
	20	0	0.032±0.001	0.017±0.001
1681.1	5	0	0.129±0.008	0.002±0.001
	10	0	0.180±0.009	0.003±0.001
	15	0	0.182±0.003	0.003±0.001
	20	0	0.189±0.003	0.002±0.001
6724.3	5	0	0.183±0.007	0.001±0.001
	10	0	0.181±0.002	0.003±0.001
	15	0	0.182±0.009	0.003±0.001
	20	0	0.193±0.009	0.003±0.001
15124.8	5	0	0.179±0.005	0.003±0.001
	10	0	0.183±0.008	0.003±0.001
	15	0	0.180±0.007	0.003±0.001
	20	0	0.221±0.008	0.003±0.001
26897.4	5	0	0.201±0.006	0.002±0.001
	10	0	0.201±0.007	0.001±0.001
	15	0	0.192±0.009	0.002±0.001
	20	0	0.190±0.002	0.003±0.001

DISCUSSION

Combination of operational parameters such as total solids, protein, lipid and alkaline phosphatase activity in supernatant, infranatant, and pellet suitable in judging the effectiveness at which insoluble are removed by various centrifugal force and centrifugation time from the Red shrimp homogenate (Shenker *et al*, 1993). RCF of 67.2×g up to 20 min was able clarify only two third of the cell debris from the homogenate that is supported by the finding of Takagi *et al*. (2000) that relative centrifugal force of 67×g for 5 min achieves less than 91% recovery of the tissues. RCF of 1681.1×g efficiently clarifies four fifth of the cell debris from the homogenate even at the end of 5 min, which is supported by the findings of Erasmus *et al*. (1997) that centrifugation of the tissue homogenate at RCF of 1089×g for 15 min was able to clarify the hepatopancreatic tissue homogenates from all the cell debris. Here, reduced force and resident time in the centrifuge would contribute partial clarification of the tissue homogenate from insoluble, and increasing the force and time may increase the sedimentation rate (Takagi *et al*., 2000, Yassien and Asfour, 2012).

RCF of 67.2×g even up to 20 min of centrifugation was able to remove less than one fifth of the total soluble protein of the homogenate. However, RCF of 1681.1×g for 5 min of the centrifugation reduced one fourth of the protein from the homogenate, and 10 min of centrifugation at this speed reduced four fifth of the protein. Beyond 10 min of centrifugation at RCF of 1681.1×g, or beyond RCF 1681.1 even up to 20 min of centrifugation was not able to remove protein from the homogenate. During the centrifugation of the homogenate at different RCF and resident time in the centrifuge, lipid present in the homogenates underwent floatation to form a fully developed layer on top of the medium. Even though RCF of 67.2×g even upto 20 min clarified less than one fourth of the lipid, RCF of 1681.1×g clarified three fourth of the lipids of the homogenate only by the end of 5 min of centrifugation, and more than 90% of the lipid at the end of 10 min of centrifugation. RCF of 1681.1×g for 10 min is the optimum centrifugation parameter the homogenate as it clarifies largest quantity of the lipids into the supernatant, retains protein of interest in the infranatant, and removes impurities into pellets (Shenker *et al*., 1993).

Even during the entire 20 min of centrifugation at RCF of 67.2×g or during 5 min of centrifugation at RCF of 1681.1×g homogenate lost less than 10% of the activity. Nagahashi and Hirike (1982) showed that low centrifugal force is efficient in maintain optimum enzyme activity. However beyond 5 min of centrifugation at RCF of 1681.1×g or beyond RCF of to 1681.1×g even at 5, homogenate lost more than half of the alkaline phosphatase activity. Enzymes in the biological fluids are subjected to fluidic forces in the centrifuge and resulting hydrodynamic sheer force may denature low molecular weight proteins (Elias and Joshi, 1998). Purification strategy based on laboratory test values is correctly made when each process of the purification stage is properly performed to improve productivity (Roush and Lu, 2008). Specific activity of the homogenate increased by 4.14±0.02

folds by 5 min of centrifugation at RCF of 1681.1×g. The incremental specific activity of the tissue homogenates remained at a constant level of 6.36±0.49 folds compared to its respective homogenate beyond 5 min at RCF of 1681.1×g or beyond RCF of 1681.1×g for 5, 10, 15 or 20 min. Optimum alkaline phosphatase activity in the homogenates was attained at RCF of 1681.1×g for 5 min.

Selecting the suitable g-force and resident time of the centrifugation important because wide variation in the physico-chemical properties and proportion of these components in biological fluids is a significant bottle-neck in primary clarification and properly (Yavorsky *et al*., 2003). Optimum alkaline phosphatase yield was achieved at RCF of 1681.1×g for 5 min at 4^{o}C, and increase in centrifugal force beyond this level was efficient in reducing the total protein content from the homogenate but the homogenate lost its alkaline phosphatase activity. Hence, inefficient centrifugation to remove insoluble may result in the complete or partial loss of the enzyme from the purification stream due to hydrodynamic sheer force of centrifugation (Elias and Joshi, 1998). This issue should be considered with special concern, as it may adversely affect purification strategy and cost effectiveness.

CONCLUSION

RCF of 1681.1×g for 5 min in the centrifugae at 4^{o}C is an efficient clarification method hepatopancreatic tissue homogenate of Tiny shrimp. The RCF below 1681.1×g at any given resident time results in the incomplete removal of lipids and cell debris from the homogenate, and the centrifugal force above 1681.1×g at any given time or resident time above 5 min at 1681.1×g results in partial loss of the enzyme from the purification stream due to hydrodynamic sheer force of centrifugation. This is an important issue as it may adversely affect purification strategy and cost effectiveness.

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