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Original Research

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Effective removal of insolubles from brown shrimp hepatopancreatic homogenate during primary stages of alkaline phosphatase recovery

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ABSTRACT

Background and Objectives: Commercially important alkaline phosphatase of novel characteristics can be isolated from the hepatopancreatic tissues of Brown shrimp (*Metapenaeus monoceros*). However, interference of insoluble released during the homogenisation may interfere with subsequent recovery steps. Hence, present study is aimed at designing an effective insoluble removal method to isolate alkaline phosphatase with optimum yield and activity. **Materials and Methods:** Hepatopancreatic tissues of the shrimp was homogenized at 3,000 rpm for 10 min at 4oC in a homogenizer and the tissue homogenates were centrifuged at Relative Centrifugal Force of 67.2, 1681.1, 6724.3, 15124.8 or 26897.4 × g for 5, 10, 15, or 20 min at 4°C. **Results:** Relative Centrifugal Force of 1681.1×g for 5 min at 4°C successfully clarified 90.28±1.55% of the insolubles. The forces below this level even upto 20 min removed only up to 56.57±0.66% of the total solids and 7.90±0.19% of the total lipids from the homogenate. Force and time above RCF of 1681.1×g for 5 min, specific activity was increased by 4.18±0.38 folds due to the loss of total protein, but upto 60.48±2.61% of alkaline phosphatase activity was lost. **Conclusion:** Hence, the force of 1681.1 × g and resident time of 5 min in the centrifuge is an efficient clarification method of tissue homogenate

KEY WORDS: Alkaline phosphatase, centrifugation, clarification, insoluble, shrimps

INTRODUCTION

Commercially important Alkaline phosphatase (EC 3.1.3.1) of unique properties of industrial importance can be isolated from the hepatopancreas of Brown shrimp (*Metapenaeus monoceros*) abundantly available along coastal Karnataka. Hepatopancreatic waste is the rich source of alkaline phosphatase [1]. Different marine organisms exposed to extreme environmental conditions are the source of enzymes adapted to these extreme environmental conditions [2]. However, recovery of such an important enzyme of unique properties from the tissues is cumbersome due to the interference of insoluble released during cell disruption [3]. Centrifugation is a popular method of clarification of insolubles when the product of interest is in the homogenate [4]. Insoluble released during the homogenization may vary in their physico-chemical properties and is proved troublesome [5-7]. Carefully selecting criterions of clarification of the homogenate using centrifugation such as the force and the resident time is important to ensure high yield of the enzyme, consistency of the output and reproducibility of the method [8]. Insufficient force and resident time in the centrifuge may reduce the enzyme yield and the extreme force and prolonged exposure in the centrifuge may denature the enzyme due to hydrodynamic sheer force [9-12]. By carefully designing the centrifugation parameters under proper laboratory conditions paved the way for the optimum productivity [13]. Lots of work has been carried out in this regard [14-16]. However, no single set of clarification parameters are standardized in terms of centrifugation force an resident time has be defined to isolate alkaline phosphatase from the hepatopancreas. Hence, present study is focused on determining relative centrifugal force and resident time to efficiently clarify alkaline phosphatase for the hepatopancreas of Brown shrimp with optimum yield 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 [17]. Buffer used for the homogenization of hepatopancreatic tissues of shrimps was 0.1 M Tris-HCl buffer of pH 8.4. However,

2-amino-2-methyl-1-propanol buffer of pH 10.3 was used as an assay buffer thought the experiment. All the buffer preparations were filtered and sterilized in moist heat at 121°C for 20 min [18].

Sample Collection

Brown shrimp caught near coastal Karnataka between the months of July and December was transported to the laboratory within 4-6 h maintained at 4°C and identified [19]. The shrimps belonging in the size group of 86-120 mm in length, and weighing around 30-55 g were washed and dissected to remove the hepatopancreas for further processing. Hepatopancreatic tissues were packed in plastic bags, labeled, frozen at −40°C, and stored at −20°C in a deep freezer (JHBio, Chennai, India) for subsequent processing.

Homogenization

The samples 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. The samples were homogenized at speed of 3000 rpm for 10 min at the temperature of 4°C using 0.1 M Tris-HCl buffer of pH 8.4 at 1:10 tissue to buffer ratio [20].

Centrifugation

The homogenate with highest protein content was centrifuged at RCF of 67.2, 1681.1, 6724.3, 15124.8 or 26897.4 ×*g* for 5, 10, 15, or 20 min at 4°C in the centrifuge (Remi Laboratory Instruments, Mumbai, India). After centrifugation, each infranatant collected was estimated for total solid content, protein content, fat content and alkaline phosphatase activity. Supernatant layer and pellets were reconstituted using 0.1 M Tris-HCl buffer of pH 8.4 at 1:10 pellets to buffer ratio and 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 [21], and expressed as percentage moisture. The protein content was estimated as per the Folin-Ciocalteu method of Lowry *et al*., [22], using bovine serum albumin as a standard. The lipids were quantitatively estimated by sulfo-phospho-vanillin method of Barnes and Blackstock [23] using ultraviolet-visible double beam spectrophotometer (Systronics, Mumbai, India).

Enzyme Assay

The procedure used for alkaline phosphatase analysis was based on the method of Bowers and McComb [24] using disodium paranitrophenyl phosphate (*p*NPP) as a substrate. Alkaline phosphatase activity in units/L is the liberation of 1 mM of *p*NP per min at 37°C incubation temperature per liter of tissue homogenate in respective buffers. We made no corrections for the slight variation of molar absorptivity of *p*NP with pH and (or) buffer concentration.

Statistical Analysis

The analysis of cell disruption was carried out in quadruplicate. The results were treated using analysis of variance (ANOVA), followed by Tukey's test, using the software Statistical 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 AND DISCUSSION

Efficiency of the removal of insoluble by various centrifugal force and resident time from the homogenate was done by estimating total solids, protein, lipid and alkaline phosphatase activity in supernatant, infranatant, and pellet obtained through combination of these operational parameters [25].

Solids Fractionation at Different Centrifugal Force and Time

The homogenates were made up of 23.67 ± 0.78% of solids. Even though RCF of 67.2 ×*g* was able to clarify 69.37 ± 0.21% of the total solids by 20 min, almost 64.06 ± 0.12% of the total solids were clarified before 5 min. Takagi *et al*., have reported to have achieved less that 91% recovery of the tissues when the centrifugal force was applied for 5 min at 67 ×*g* [10]. Whereas, RCF of 1681.1 ×*g* was able to clarify 87.89 ± 0.02% of the total solids by 5 min, and 97.07 ± 0.33% of the total solids by 10 min of centrifugation from the tissue homogenates. Erasmus *et al*., had reported that centrifugation at RCF of 1089 ×*g* for 15 min removed all the cell debris from the hepatopancreatic tissue homogenates [26]. However, one-way ANOVA with *post-hoc* Tukey's test, was not able to establish any significant ($P > 0.05$) difference in the solid content among 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]. Reduced centrifugal force and time would contribute partial isolation of insoluble from the homogenate, and increasing RCF and resident time may increase the sedimentation rate [10,27].

Protein Fractionation at Different Centrifugal Force and Time

Total protein estimated in the homogenate was 5775.00 ± 150.00 mg/L. Centrifugation of the homogenate even up to 20 min at RCF of 67.2 ×*g* was able to fractionate only 19.73 ± 0.67% of the total soluble protein into pellets. Whereas, RCF of 1681.1 ×*g* fractionated 68.25 ± 0.97% of the

protein by 5 min, and 92.31 ± 0.94% of the protein by 10 min of centrifugation. One-way ANOVA with *post-hoc* Tukey's test was not able to establish any significant ($P > 0.05$) difference in the protein content among 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 [Table 2]. No protein accumulation ($P > 0.05$) was registered in the supernatant all along these period at any centrifugal force.

Lipid Fractionation at Different Centrifugal Force and Time

The homogenate was estimated to have 3667 ± 28.42 mg/L of lipid. During centrifugation at different force and time interval total lipid present in the initial homogenates underwent floatation to form a fully developed layer on top of the medium [25]. RCF of 67.2 ×*g* was able fractionate only 17.62 ± 0.07% of the lipids of the initial homogenate to the supernatant even at the end of 20 min of centrifugation. However, RCF of 1681.1 ×*g* was able to reduce 67.41 ± 0.64% of lipid by 5 min, and 92.11 ± 0.12% of the lipid from the homogenate at the end of 10 min of centrifugation. However, subsequent 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 ($P > 0.005$) any lipid content in the homogenate or the supernatant, as estimated by one-way ANOVA with *post-hoc* Tukey's test [Table 3].

Fractionation of the Enzyme Activity at Different Centrifugal Force and Time

During the entire 20 min of centrifugation at RCF of 67.2 ×*g*, the homogenate lost only 6.96 ± 4.39% enzyme activity, and specific activity remained at a level of 0.02 ± 0.01 units/mg [Tables 4 and 5]. Whereas, RCF of 1681.1 ×*g* was able to retain 96.66 ± 1.79% of the activity in the infranatant at the end of 5 min and during this period specific activity increased by 3.04 ± 0.01-fold. Nagahashi and Hiraike reported that low centrifugal force is efficient in maintain optimum enzyme activity and increase in speed and time does not have any effect on the activity [28]. However, when the resident time was increased to 10 min at RCF of to 1681.1 ×*g*, the activity was only 45.59 ± 1.34% of its respective initial homogenate. During centrifugation of biological fluids proteins, especially enzymes are subjected to fluidic forces and resulting hydrodynamic sheer force may cause damage to the low molecular weight proteins, resulting in denaturation and inactivation of protein [12]. Here, at and beyond 10 min at RCF of 1681.1 ×*g* or beyond RCF of 1681.1 ×*g* even at 5 min resulted in significant ($P < 0.05$) loss in the activity.

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 selecting the g-force and resident time of the centrifugation affect the efficiency of the centrifuge [5,6]. Optimum yield of the enzyme achieved at RCF of 1681.1 ×*g* for 5 min at 4°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 [11,12]. This issue should be considered with special concern, as it may adversely affect purification strategy and cost effectiveness.

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

RCF (×*g*)	Resident time (min)	Solids (%)		
		Supernatant	Infranatant	Pellets
67.2	5	0.12	8.99	15.89
	10	0.36	8.37	16.26
	15	0.49	8.06	16.47
	20	0.65	7.66	16.72
1681.1	5	2.47	3.03	19.57
	10	3.38	0.73	20.89
	15	3.38	0.73	20.98
	20	3.41	0.65	21.03
6724.3	5	3.39	1.59	20.90
	10	3.39	0.70	20.91
	15	3.40	0.67	20.93
	20	3.41	0.65	21.03
15124.8	5	3.40	0.68	20.92
	10	3.41	0.66	20.93
	15	3.42	0.62	20.96
	20	3.43	0.60	21.06
26897.4	5	3.40	0.67	20.93
	10	3.44	0.57	20.99
	15	3.44	0.57	20.99
	20	3.42	0.56	21.05

RCF: Relative centrifugal force

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

RCF (×*g*)	Resident time (min)	Protein content (mg/L)		
		Supernatant	Infranatant	Pellets
67.2	5	0	5440.25±65.99	334.75±34.55
	10	0	5069.25±54.16	705.75±33.55
	15	0	4881.00±36.85	894.00±23.55
	20	0	4635.75±62.94	1139.25±33.56
1681.1	5	0	1833.75±65.86	3941.25±35.56
	10	0	444.25±65.12	5330.75±52.53
	15	0	444.25±51.76	5330.75±52.55
	20	0	394.25±53.45	5380.75±22.55
6724.3	5	0	428.75±29.51	5346.25±35.55
	10	0	421.25±50.53	5353.75±35.35
	15	0	408.00±59.75	5367.00±33.65
	20	0	391.50±50.15	5383.50±55.36
15124.8	5	0	410.50±55.45	5364.50±33.35
	10	0	399.75±54.58	5373.25±34.55
	15	0	373.75±30.52	5401.25±55.58
	20	0	365.00±55.59	5401.25±26.53
26897.4	5	0	405.50±37.58	5369.50±55.52
	10	0	348.50±15.53	5427.00±25.56
	15	0	347.00±57.55	5427.75±45.29
	20	0	340.00±21.05	5435.00±35.35

RCF: Relative centrifugal force

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

RCF (×*g*)	Resident time (min)	Lipid content (mg/L)		
		Supernatant	Infranatant	Pellets
67.2	5	122.04±03.27	3544.96±35.65	0
	10	363.79±05.47	3303.21±53.63	0
	15	486.46±03.77	3180.54±23.35	0
	20	646.27±07.43	3020.73±33.35	0
1681.1	5	2472.10±74.54	1194.90±32.32	0
	10	3377.52±33.55	289.48±05.54	0
	15	3377.52±55.55	289.48±06.66	0
	20	3410.10±56.45	256.90±05.46	0
6724.3	5	3387.62±55.54	279.38±04.55	0
	10	3392.51±15.14	274.49±14.25	0
	15	3401.14±44.65	265.86±15.35	0
	20	3411.89±44.44	255.11±43.24	0
15124.8	5	3399.51±54.24	267.47±43.53	0
	10	3406.52±45.54	260.48±45.65	0
	15	3423.46±45.42	243.04±03.25	0
	20	3429.16±43.46	237.04±02.55	0
26897.4	5	3402.77±44.33	264.03±03.75	0
	10	3440.24±43.23	226.86±07.51	0
	15	3440.73±44.64	226.77±05.09	0
	20	3445.45±44.94	221.05±07.77	0

RCF: Relative centrifugal force

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

RCF (×*g*)	Resident time (min)	Alkaline phosphatase activity (units/L)		
		Supernatant	Infranatant	Pellets
67.2	5	0	79.79±02.35	2.63±00.09
	10	0	79.34±04.35	2.63±00.13
	15	0	74.00±03.41	2.25±00.03
	20	0	73.00±07.33	3.25±00.06
1681.1	5	0	79.50±03.59	2.25±00.09
	10	0	37.50±01.51	4.75±00.07
	15	0	37.50±12.51	4.75±00.17
	20	0	41.50±02.42	4.50±00.05
6724.3	5	0	43.50±03.52	4.50±00.14
	10	0	43.75±03.53	3.75±00.05
	15	0	35.50±13.53	5.88±00.14
	20	0	39.75±01.13	4.75±00.04
15124.8	5	0	44.50±01.99	5.00±00.29
	10	0	34.00±03.50	4.00±00.14
	15	0	31.63±03.38	2.75±00.15
	20	0	32.75±03.53	5.50±00.02
26897.4	5	0	40.63±03.03	4.00±00.07
	10	0	33.00±05.72	3.00±00.05
	15	0	30.25±04.99	4.00±00.01
	20	0	29.50±04.32	3.00±00.07

RCF: Relative centrifugal force

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

RCF (×*g*)	Resident time (min)	Specific activity (units/mg)		
		Supernatant	Infranatant	Pellets
67.2	5	0	0.0147±0.002	0.0078±0.001
	10	0	0.0157±0.002	0.0037±0.001
	15	0	0.0152±0.002	0.0025±0.001
	20	0	0.0157±0.005	0.0028±0.001
1681.1	5	0	0.0434±0.002	0.0006±0.001
	10	0	0.0844±0.003	0.0009±0.001
	15	0	0.0844±0.005	0.0009±0.001
	20	0	0.1053±0.002	0.0008±0.001
6724.3	5	0	0.1015±0.002	0.0008±0.001
	10	0	0.1039±0.004	0.0007±0.001
	15	0	0.0870±0.004	0.0011±0.001
	20	0	0.1015±0.005	0.0008±0.001
15124.8	5	0	0.1084±0.004	0.0009±0.001
	10	0	0.0851±0.005	0.0007±0.001
	15	0	0.0846±0.004	0.0005±0.001
	20	0	0.0897±0.004	0.0010±0.001
26897.4	5	0	0.1002±0.004	0.0007±0.001
	10	0	0.0948±0.002	0.0005±0.001
	15	0	0.0871±0.004	0.0007±0.001
	20	0	0.0868±0.002	0.0004±0.001

RCF: Relative centrifugal force

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