FRACTIONATION OF PROTEASES FROM COWTAIL RAY (*Trygon sephen*) VISCERA USING POLYACRYLIC ACIDS

Achmad Poernomo*)

ABSTRACT

Fractionation of proteases of cowtail ray (*Trygon sephen*) viscera using polyacrylic acids has been investigated. The viscera were ensiled using a mixture of propionic and formic acids (1:1, v/v) at a level of 3% (v/w) at 40°C for 5 days. Fractionation was carried out step wise at 4°C to produce highest possible activity of the enzymes at respective fractions. It was shown that an addition of 2% polyacrylic acids (PAA) solution to produce 0.275% PAA in the final solution followed by pH adjustment to pH 4.0 of fraction containing acidic protease, and to pH 8.0 of that containing alkaline protease has been able to separate alkaline and acidic proteases. The process also purified the enzymes by 1.1 and 4.7 fold for acidic and alkaline proteases, respectively.

KEYWORDS: cowtail ray, ensilation, fractionation, polyacrylic acids, proteases

INTRODUCTION

Many methods have been studied and applied to separate proteins in solution from one another, and precipitation using polyelectrolytes is a method that is frequently used for such purpose. This technique is considered fast and safe especially to maintain the biological activity of proteins such as enzymes.

Charged polymers have been used by several workers to precipitate proteins from solution, and at the same time fractionate protein from other proteins or compounds. This method has been reported able to separate amyloglucosidase from transglucosidase (Sternberg, 1972; 1975), lipoxygenase from b-amylases (Sternberg, 1976), alkaline from acidic proteases of salmon viscera (Reece, 1988), lysozyme from egg white (Fischer & Glatz, 1988), as well as in precipitating trypsin from a pepsin-trypsin mixture (Sternberg & Hershberger, 1973), plant proteases (Caygill *et al.*, 1983), chitosanase (Boucher *et al.*, 1992) and lysozyme (Sternberg & Hershberger, 1973; Fischer & Glatz, 1988; Shieh, 1989). The charged polymers commonly used are carboxymethyl cellulose, methacrylate polymers, polyethyleneimine (PEI) and polyacrylic acids (PAA).

Cowtail ray (*T. sephen*) viscera contain several different enzymes, some of which belong to proteases group. Ensilation has proved able to extract the enzymes (Poernomo, 1998), and separation of individual enzymes is necessary since it will assist the purification of the enzymes, especially if the enzymes have different characteristics, such as those from fish viscera. This paper deals with the fractionation of alkaline and acidic proteases of cowtail ray viscera silage using PAA solution to facilitate subsequent purification and characterisation of the enzymes.

MATERIALS AND METHODS

Cowtail ray (*T. sephen*) viscera were obtained from fish drying processors at Labuhan Maringgai, Lampung, Sumatera. Separation and washing of viscera were carried out at the processing sites. The viscera were road transported in ice (1:1, w/w) to the laboratory of the Slipi Research Station for Marine Fisheries, Jakarta which took about 8-10h. Upon arrival, the viscera were once more washed in fresh water, frozen and stored at -45°C until used.

Enzymes extraction was conducted by ensilation of the viscera. Chopped (ca. 1x1 cm) viscera was added with a mixture of propionic and formic acids (1:1, v/v) at a level of 3% (v/w), and kept at 40°C for 5 days during which the mixture was stirred daily. The silage was then centrifuged at 10,000x*g* for 10 min. The sediment was separated from the silage, while the lipid on the surface was skimmed off. The supernatant was frozen (-45°C) in a chest freezer overnight and was stored at -25° to -30°C until used.

Before used, the silage supernatant was thawed in running water and centrifuged (10,000x*g*, 10 min) at 4°C and then added with 1/3 volume of ice-

*) Researcher of the Central Research Institute for Fisheries, Jakarta

cold chloroform to remove the remaining fat. Afterward, the mixture was left at 4°C for 15 min, then the lower layer was separated using a separating funnel and finally recentrifuged to remove the remaining chloroform and fat. After centrifugation, the lower layer was separated and referred to as *crude enzyme extracts*.

The alkaline and acidic proteases were fractionated with PAA solution (MW 3-5 million Dalton/MD, Carbopol C934, BF-Goodrich) through a series of preliminary trials at 4°C. A volume of crude enzyme extract was mixed with 2% (w/v) PAA solution to produce various concentrations (0.0125 to 0.3%) of PAA in the final mixture. The mixtures were stirred for 30 min and then centrifuged (10,000 x *g*, 10 min). The supernatant (S_1) was separated and referred to as *acidic protease extracts*.

The precipitates were redissolved in 0.4M Tris buffer pH 8.5 with an addition of 1M $CaCl_2$ to produce 50 mM in the final mixture. The mixture was stirred for 10 min and recentrifuged. The subsequent supernatant (S_2) was separated and referred to as *alkaline protease extracts*.

The PAA concentration producing the highest acidic protease activity and lowest alkaline protease activity in S_1 and lowest acidic protease activity and highest alkaline protease activity in S_2 was applied in the next trial. In the second trial, S_1 was adjusted to pH 4.0, pH 3.5 and pH 3.0, while S_2 was adjusted to pH 8.5 and pH 9.0 using 2N HCl or 2N NaOH, respectively. Schematically, the process is illustrated in Figure 1.

Protease activity was monitored by the method of Barret (1972) modified by Martinez *et al.* (1988). Universal buffers of pH 3 and 8 (Johnson & Lindsey 1939) were used as assay buffers and for enzyme dilution. Alkaline protease was monitored at pH 8 using 8% casein in 50 mM NaOH, while acidic protease was monitored at pH 3 using 8% hemoglobin in deionised water.

To a mixture of 0.5 mL assay buffer and 0.25 mL substrate equilibrated at 25°C was added 0.25

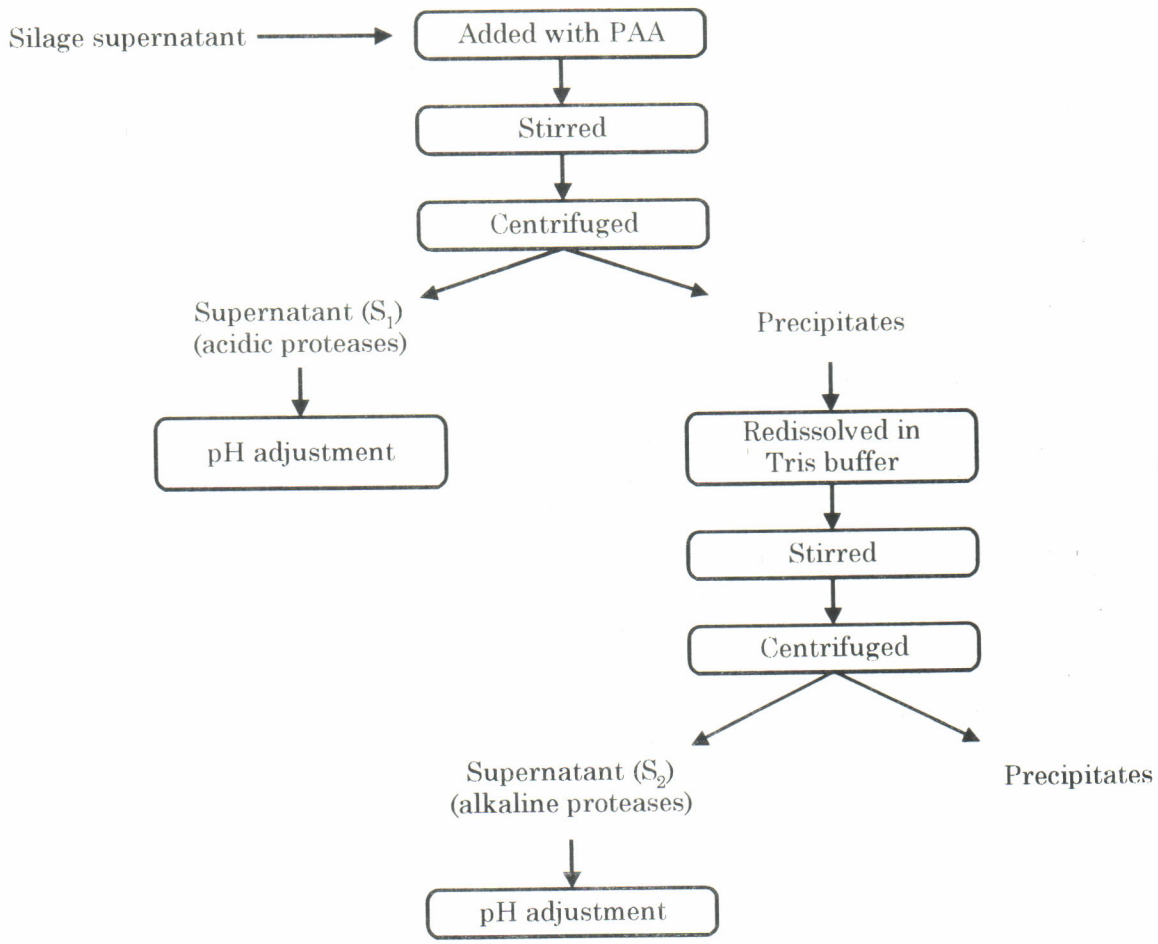

Figure 1. Fractionation of alkaline and acidic proteases using polyacrylic acids (PAA).

mL enzyme solution. After 60 min incubation at 25°C, 5 mL 6% TCA was added to stop the reaction. Filtration was done after 15 min using Advantec filter paper no. 131 (equivalent to Whatman filter paper no. 3). The absorbance of the filtrate was read at 280nm and was corrected for the respective blanks. Blanks were obtained in a similar manner, but TCA was added before the enzyme. One unit of enzyme activity was defined as the increase of absorbance by 1.0 at 280 nm of TCA soluble material in 60 min at 25°C.

RESULTS

The results of fractionation of alkaline and acidic proteases are presented in Figure 2 and Table 1. In the first trial (Figure 2), acidic proteases always remained in S_1 at levels of more than about 80% of the original and precipitated (contained in S_2) at levels lower than 5%. Alkaline proteases, however, remained in S_1 at levels higher than those in the precipitate (in S_2) for PAA final concentrations below 0.1%. At final concentrations higher than 0.1%, the alkaline protease activities were higher in the precipitate (in S_2) than in S_1.

A PAA final concentration of 0.2% resulted in the highest acidic protease activity in S_1 (about 98%). However, at this concentration, the supernatant still contained about 30% alkaline protease activity, while in S_2, the alkaline protease activity was still low (about 50%) although the acidic protease activity was also low (about 3%). At a PAA final concentration of 0.275%, highest alkaline protease activity (about 65%) in S_2 was obtained. At this concentration, about 80% of acidic protease activity was retained in S_1 while the alkaline protease activity in S_1 was the lowest. This treatment was used in the subsequent fractionation step.

The pH adjustment of S_1 and S_2 was to inactivate the alkaline protease in S_1 and acidic protease

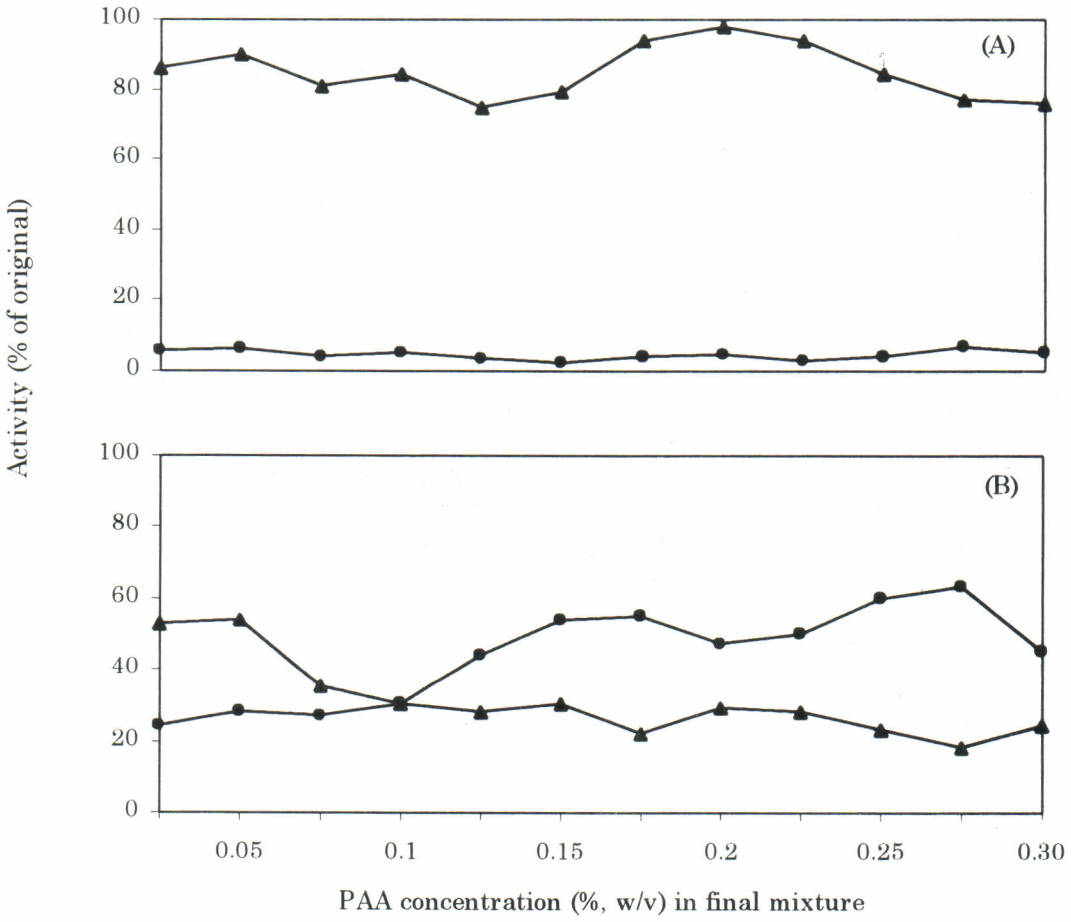

Figure 2. Distribution of acidic and alkaline proteases activity after PAA addition
A = Acidic proteases; B = Alkaline proteases; ▲ = Supernatant (S_1); ● = Precipitate (S_2)

Table 1. Distribution (%) of acidic and alkaline proteases during fractionation with PAA and pH adjustment

Fractionation step	Supernatant (S_1)		Precipitate (S_2)	
	Acidic activity*	Alkaline activity*	Acidic activity*	Alkaline activity *
PAA **	77.9 ± 3.8	6.1 ± 0.3	19.8 ± 0.2	60.8 ± 2.0
pH adjustment:***				
9.0	-	-	0	57.4 ± 1.9
8.5	-	-	0	63.1 ± 1.4
4.0	99.5 ± 2.0	0	-	-
3.5	76.0 ± 1.7	0	-	-
3.0	12.1 ± 0.7	0	-	-

* % of original, values are means ± standard deviation for 2 replicates, each from 3 determinations. 1 enzyme unit was defined as the increase of the absorbance at 280 nm of TCA soluble material in 60 min at 25°C using casein or hemoglobin as substrate

** Final concentration is 0.275

*** Using 2N HCl or NaOH

in S_2. As shown in Table 1, the pH adjustment of S_2 did not significantly change the alkaline protease activity. However, the acidic protease activity of this fraction was eliminated. The alkaline protease activity in S_2 was zero after pH adjustment, but the acidic protease activity declined as the pH was reduced to 3.5 (76% remained) and 3.0 (12.1% remained). At pH 4.0, the acidic protease activity increased to 99.5% of the original.

Fractionation also partly purified the enzymes, and in a separate study it was shown that fractionation steps such as those in this study have been able to purify the enzymes by 1.1 and 4.7 fold for acidic and alkaline proteases, respectively (Table 2).

DISCUSSION

The specific activity of alkaline protease in the supernatant of cowtail ray viscera silage, as shown in Table 1, was lower than that of salmon viscera silage as reported by Reece (1988). This was probably due to the differences in species as well as the procedure of ensilation. Moreover, cowtail ray is an elasmobranch fish which does not have a distinct pyloric caeca as a source of alkaline proteases. The period of silage storage in the present study was 5 days at 40°C and this might have affected the stability of alkaline protease in acidic conditions. Reece (1988) further reported that alkaline proteases of cod and mackerel viscera were not stable under acidic conditions during ensilation and only acidic proteases were recovered.

In the present study, PAA was used since it has been used by Sternberg & Hershberger (1973), Caygill *et al.* (1983) and Reece (1988) to successfully fractionate alkaline and acidic proteases. In addition, in the preliminary study, the use of another polyelectrolyte, PEI, to precipitate acidic proteases proved unsatisfactory. In the precipitation of protein by PEI, salt must be added to aid the process (Bell *et al.*, 1983; Jendrisak, 1987) and salt was found to affect enzyme activity (Thongthai *et al.*, 1990; Gildberg, 1993; Gildberg *et al.*, 1991; Xu *et al.*, 1996).

The use of low molecular weight (90,000D) PAA was also tested in the preliminary study. However, the results were not satisfactory since the contamination of acidic proteases was high in the precipitate. Although low molecular weight (90,000-250,000D) PAA has been successful in precipitating some enzymes (Sternberg & Hershberger, 1973; Sternberg, 1976; Shieh & Glatz, 1991; Boucher *et al.*, 1992), it seems that low molecular weight PAA was not suitable for fractionation of alkaline and acidic proteases from cowtail ray viscera. In the fractionation of transglucosidase and amyloglucosidase (Sternberg, 1975) PAA of at least 1MD molecular weight was recommended. According to Sternberg (1976) the formation of an insoluble protein-polyacrylate complex depends among others on the molecular weight of PAA and the ratio of PAA/

Table 2. Purification of alkaline and acidic proteases of cowtail ray viscera by fractionation.

Purification steps (Fraction)	Alkaline proteases					Acidic proteases				
	Total protein (mg)	Total enzyme activity (D A)*	Specific activity (D A/mg)	Recovery (%)	Purification (fold)	Total protein (mg)	Total enzyme activity (D A)*	Specific activity (D A/mg)	Recovery (%)	Purification (fold)
Crude silage	7223.4	486	0.07	100	1	7223.4	1949.4	0.27	100	1
Defatted silage	7287.3	498.4	0.07	100	1	7287.3	1829.8	0.25	93.9	0.9
PAA										
S_1**	-	-	-	-	-	6285.4	1783	0.28	91.5	1.1
S_2**	910.7	298.4	0.33	61.4	4.7	-	-	-	-	-

* one enzyme unit was defined as the increase of the absorbance at 280 nm of TCA soluble material in 60 min at 25^0C using casein or hemoglobin as substrate

** See text for detailed explanation

protein. Higher molecular weight PAA was then tested in the present study.

Using PAA of 3-5MD molecular weight, the precipitation pattern of the alkaline protease, which was indicated by the enzyme activities, was asymmetrically bell shaped (Figure 2). A similar result was also shown by Caygill *et al.* (1983) for plant proteases (papain and ficin) and Boucher *et al.* (1992) in precipitating *Streptomyces* N174 chitosanase. It was suggested that the decrease in recovery at higher polyelectrolyte dosage was due to the excess polyelectrolyte which was incorporated into the already formed protein-polyelectrolyte complex, resulting in a soluble complex with a significant net charge, and thus hindering the aggregation of the complexes (Caygill *et al.*, 1983; Clark & Glatz, 1987, 1988; Chen & Berg, 1993).

In their studies, Caygill *et al.* (1983) employed PAA/protein ratios of 0.02-0.5, while Boucher *et al.* (1992) used ratios of up to 10. The optimum ratio for plant proteases was between 0.14-0.29 and 0.2-0.4 for papain and ficin, respectively, while for chitosanase it was around 4. In the present study, with final concentration of PAA in the mixture ranging from 0.025 to 0.3%, or a PAA/protein ratio of 0.006 to 0.075 the optimum ratio to achieve the highest alkaline protease activity in the precipitate was 0.069. These ratios were much lower than those of the above studies which could be due to the different natures of the proteases as well as the PAA. Boucher *et al.* (1992) used PAA with molecular weight of 250,000D, while the one used in the present study was 3-5MD. The molecular weight of PAA used by Caygill *et al.* (1983) was not reported.

A similar ratio of PAA/protein (0.066) was used by Reece (1988) to separate alkaline protease from acidic protease of salmon viscera silage. No investigation of the optimum ratio was reported in Reece's study. However, it was reported that the alkaline protease activity in the precipitate was 82.7% of the original, while that of acidic proteases in the supernatant was 100%. The data for the acidic protease in Reece's study were reported to be modified to take into account of the co-purification of the alkaline protease, however, no explanation of the modifications were mentioned. No data on the activity of alkaline protease in the supernatant or acidic protease in the precipitates were reported. PAA precipitation in Reece's study increased the purity of the acidic and alkaline proteases by 1.6 and 6.8 fold, respectively, while in the present study, activities were 1.1 and 4.7 fold, respectively.

CONCLUSION AND RECOMMENDATION

This study showed that polyacrylic acids were able to fractionate proteases from cowtail ray viscera and at the same time increased the purity of the enzymes. It was demonstrated that the optimum fractionation condition was an addition of 2% (w/v) PAA to produce 0.275% in the final mixture, followed by pH adjustment of supernatant containing acidic protease to pH 4.0, and of that containing alkaline protease to pH 8.5. This treatment increased the specific activity of alkaline and acidic proteases by 4.7 and 1.1 fold, respectively. Practical protocol on the fractionation is necessary to be developed so that the procedure can be applied in higher scale of fractionation. Further purification following fractionation needs to be investigated so that the enzymes can be properly characterized.

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