METHODOLOGY

3.0 METHODOLOGY

Proteases constitute the most important group of industrial enzymes used in the world today and have several applications in the food industry (El-Beltagy *et al.*, 2004). The ability of any fish to digest a given diet and absorption of its nutrients depends on the presence and quality of digestive enzymes. Although several on-farm feeds are in use in aquaculture, commercial fish feeds manufactured on the basis of proper understanding of digestive physiology of fish are yet to be established for most of the cultured species [Seenappa and Devaraj, 1995]. As protein utilization is fundamental to growth, proteases have an important role to play in the larval fish as in the adult. Fish processing wastes including viscera have been reported to be good sources of protein including enzymes and fats (Arnsen and Gildberg 2007), good substrate for lactic acid fermentation (Gao *et al.*, 2006) and source of protease producing bacteria (Bhaskara *et al.*, 2006). If these biological compounds can be recovered, it would serve the dual purpose of recovery of these biomolecules and reducing the pollution problems associated therewith. Hence, the present study entitled "Comparison of various Precipitation Techniques and Partial Purification of Protease from the Visceral Organ Wastes and Head and Tail wastes of Indian oil Sardine (*Sardinella longiceps*) Fish" was taken up with the view of utilizing the large amounts of fish waste for the extraction of a beneficial enzymes, namely, proteases.

The experimental procedures for the above are discussed under the following headings:

3.1 SELECTION AND COLLECTION OF FISH WASTES

3.2 PREPARATION OF CRUDE HOMOGENATE

3.3 PRECIPITATION OF PROTEINS

3.1 SELECTION AND COLLECTION OF FISH WASTES

A survey was conducted in various fish markets of Coimbatore to find out the different kinds of fish consumed by the population. One of the most commonly consumed fish species was found to be the Indian oil sardine (*Sardinella longiceps)* commonly called "Matthi" in Tamil and Malayalam. Hence the visceral organ and head and tail wastes of this fish were collected soon after cutting the fish. The wastes were maintained in ice and brought to the place of study. After that, they were thoroughly washed in tap water and finally in distilled water and stored in a deep freezer until use.

3.2 PREPARATION OF CRUDE HOMOGENATE

The fish visceral organ and head and tail wastes were weighed and cut into small pieces with a sterile blade. These pieces were homogenized with phosphate buffered saline (0.2 M) of pH 7.4 and the homogenate was centrifuged at 10,000 rpm for 10 minutes at 4°C to obtain a clear supernatant. The procedure for this is described in Appendix I.

3.3 PRECIPITATION OF PROTEINS

3.3.1 AMMONIUM SULPHATE FRACTIONATION OF PROTEASE

Ammonium sulphate fractionation was done with the crude supernatant to get pellets of protease enzyme. Various concentrations of ammonium sulphate were added, that is, from 10% to 100%. The ammonium sulphate was added to aliquots of the crude supernatant enzyme and stirred slowly for an hour at 0°C. The mixture was then centrifuged at 4°C at 5000 rpm for 20 minutes and the pellet collected. The procedure for the above is given in Appendix II.

3.3.2 ACETONE PRECIPITATION OF PROTEASE

Acetone precipitation of protease was done with the crude supernatant to get pellets of protease enzyme. It was done with various concentrations of acetone ranging from 10% to 100% and stirred slowly. The mixtures were centrifuged at 5,000 rpm for 20 minutes at 4°C and the pellets collected. The protocol for this is given in Appendix III.

3.3.3 ETHANOL PRECIPITATION OF PROTEASE

Ethanol precipitation was done with the crude supernatant, to get pellets of the protease enzyme. Varying concentrations of ethanol ranging from 10% to 100% and were added to the crude supernatant and stirred slowly. The mixtures were centrifuged at 5,000 rpm for 20 minutes at 4°C and the pellets collected. The procedure for this is described in Appendix IV.

3.4 PURIFICATION OF PROTEASE BY DIALYSIS

The enzyme isolated from the fish visceral organ and head and tail wastes were dialyzed against phosphate buffered saline (PBS) as explained in Appendix V.

3.5 PURIFICATION OF PROTEASE BY GEL FILTRATION CHROMATOGRAPHY.

The dialyzed enzyme samples were fractionated on a Sephadex G-100 column. The column was equilibrated and eluted with 0.2M phosphate buffered saline, pH 7.4. Appendix VI gives the procedure for the same.

3.6 ESTIMATION OF PROTEIN CONTENT

The protein contents of the crude, ammonium sulphate precipitated, ammonium sulphate precipitated + dialysed, ammonium sulphate precipitated +Sephadex G-100 run, acetone precipitated, acetone precipitated + dialysed, acetone precipitated +Sephadex G-100 run, ethanol precipitated, ethanol precipitated + dialysed and ethanol precipitated +Sephadex G-100 run were determined by the Method of Lowry *et al.*, (1951). The protocol for this is explained in Appendix VII.

3.7 ASSAY OF PROTEASE

The protease activity in the crude, ammonium sulphate precipitated, ammonium sulphate precipitated + dialysed, ammonium sulphate precipitated +Sephadex G-100 run, acetone precipitated, acetone precipitated + dialysed, acetone precipitated +Sephadex G-100 run, ethanol precipitated, ethanol precipitated + dialysed and ethanol precipitated + Sephadex G-100 run were colorimetrically assayed using casein as the substrate. The method for this is explained in Appendices VIII.

3.8 STATISTICAL ANALYSIS

The data obtained from the above study was subjected to statistical analysis (One way ANOVA and Two way ANOVA) to find out the significant differences among the parameters studied.

The findings of the study are presented and discussed in the following chapter.

Plate I

Indian oil sardine (*Sardinella longiceps*)

Plate II

Viseral organ and Head and tail wastes of Indian oil sardine

(*Sardinella longiceps*)