THE PHYSICAL CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF HERRING PROTAMINES

ANNETTE JUNE MORGAN CLARKE

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THE PHYSICAL CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF HERRING PROTAMINES

BY

Annette June Morgan Clarke B.Sc (Hon.)

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Department of Biology
Memorial University of Newfoundland

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ABSTRACT

Protamines are a class of low molecular weight highly basic proteins found in the sperm nuclei of a variety of animals. These proteins are often heterogeneous with components that differ slightly in amino acid sequence. According to published data, herring protamine (or clupeine) can be fractionated into 3 components, namely clupeine Y-I, Y-II and Z, via ion exchange chromatography. The amino acid compositions and sequences of these 3 components have also been published.

In this study, protamines were isolated from the gonads of herring, *Clupea harengus*, and compared with commercially available herring protamine (Sigma clupeine). Protamines were first extracted from mature herring testes of uncertain stage in spermatozoan development. The protamine yield was low, therefore another extraction was performed on sexually mature, or ripe gonads and a much higher amount of protamine was obtained. Ion exchange chromatography suggested that the first protamine extract contained 9 components (labelled A through I), while the extract from ripe gonads had 3 major components (labelled MA, MB and MC). The chromatogram of commercial clupeine also showed 3 components (labelled SA, SB, and SC). Despite the presence of multiple components in these protamine extracts, both the intact protamines and the components fractionated by chromatography each gave only a single protein band after polyacrylamide gel electrophoresis and subsequent protein staining. After SDS-PAGE and protein staining, there was no band observable for whole Sigma clupeine. However, salmon protamine

(Sigma) and protamine extracted from ripe herring testes each had a single protein band. Protamines extracted from herring testes of uncertain stage in spermatozoan development gave 4 bands. The amino acid compositions of the fractionated components of isolated protamine, MA, MB and MC, and Sigma clupeine, SA, SB, and SC, correspond well with the published amino acid sequences of clupeine Y-II, Y-I, and Z respectively. The molecular weights obtained by electrospray mass spectrometry of fractions MA, MB, MC, SA, SB and SC are higher than those predicted from the amino acid compositions and from the published molecular weights of clupeine Y-II, Y-I and Z. This suggests that either the original sequences are incomplete or that microheterogeneity exists in these fractions. Whole protamine from ripe gonads and whole Sigma clupeine were also tested for their antimicrobial activity using a broth dilution assay. Both were found to inhibit the growth of the Gram-positive and Gram-negative organisms tested.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Terms

ATCC - American Type Culture Collection

CFU - colony forming units

Da - daltons

g - gram

g - force due to gravity

LCDC - Laboratory Centres for Disease Control

M - molar

mA - milliamps

MUN - Memorial University of Newfoundland

mwco - molecular weight cut-off

PAGE - polyacrylamide gel electrophoresis

psi - pounds per square inch

R_f - relative mobility

RT - room temperature

V - volts

Chemicals

Bis - N',N'-methylene-bis-acrylamide

BSA - bovine serum albumin

$CuSO_4 \bullet 5H_2O$ - copper sulfate pentahydrate

Na_2CO_3 - sodium carbonate

NaEDTA - ethylenediaminetetraacetic acid (disodium salt)

NaOH - sodium hydroxide

PMSF - phenylmethylsulfonyl fluoride

SDS - sodium dodecyl sulfate

TEMED - N',N',N',N'-tetramethylethylenediamine

Tris - tris(hyroxymethyl)aminomethane

Media

MHB - Mueller-Hinton broth

TSA - trypticase soy agar

LEB - *Listeria* Enrichment Broth

1. INTRODUCTION

Protamines are low molecular weight, highly basic proteins found in combination with DNA in the nuclei of sperm cells from a variety of animals. These proteins displace histones at the terminal stage of spermatogenesis and bind to nucleosomal DNA to form nucleoprotamine which is very compact and transcriptionally inactive (Okamoto *et al.*, 1993).

1.1 History

The first protamine was isolated by Meischer (1874) during his research on cellular nuclei. He found, in the sperm of Rhine salmon, a nitrogenous base combined with what he termed "nuclein" , what we now call nucleic acid. The nitrogenous base, which occurred in a salt-like linkage with the nucleic acid and could be precipitated as a salt using platinum chloride, he named "protamine" . In 1896, Kossel obtained amino acids upon hydrolysis of a similar base he had isolated from sturgeon, and thus determined the protein nature of these compounds. He proposed that the term protamine, introduced by Meischer, be used as a general name for these proteins and that individual protamines should be named after the family, genus or species of fish that they were isolated from (Kossel, 1896 a, b). For example clupeine is from herring, *Clupea*. This naming system was followed for a number of decades but is not consistently used today (Oliva and Dixon, 1991). Kossel, and his co-workers, continued to isolate protamines from a number of different fish species and also developed methods of separating and quantifying the three basic amino acids (arginine, lysine, and histidine) after protamine hydrolysis (Kossel, 1928).

This marked the beginning of the characterization and analysis of the structure of protamine. Believing that research into these simpler proteins would provide important information about proteins in general, groups of German researchers led by Kossel, Felix, and Waldschmidt-Leitz continued their study and built up a general view of protamines. Their work, over a period of 50 years, laid the foundations of protein chemistry. However, further research into the exact structure and amino acid sequence of these proteins was hindered by the technology of the day, the heterogeneity of the protamine, and the number of arginine residues in a protamine molecule (Ando *et al.*, 1973). Protamines, like other proteins, were typically analyzed by partial hydrolysis and subsequent piecing together of the resultant peptide fragments. Because of the number of successive arginine residues and the presence of multiple similar components, the correct order of these fragments was very difficult if not impossible to determine with certainty for protamines (Felix, 1960). Around the time of World War II, great advances were made in chemical methods and techniques which made it possible for researchers to overcome many of the difficulties. In Tokyo, Ando and his colleagues were able to isolate homogeneous components of clupeine and determine their amino acid sequences. Their studies established a general method of determining the primary structure of each protamine component (Ando *et al.*, 1973). Since then, a number of fish protamines have been purified and sequenced.

Since the 1950's protamines have been isolated from the sperm nuclei of many organisms other than fish. The first such report was from Daly *et al.* (1951) who isolated the protamine Galline from fowl (*Gallus domesticus*). Over

the next 20 years, protamines were isolated from earth worms, insects, gastropods, amphibians, reptiles, birds, marsupial and placental mammals, and even some plants such as the club moss and liverwort. These are described in reviews by Bloch (1969,1976).

1.2 Classification

Protamines are a member of a group of basic proteins found complexed with DNA in sperm nuclei. In contrast to the evolutionary conservatism of somatic histones, the basic proteins of sperm are quite diverse (Bloch, 1969). This group of proteins has been classified in a number of ways. Kossel (1928) grouped the fish protamines according to their content of the three basic amino acids arginine, lysine and histidine. Monoprotamines, for example salmine, clupeine, and salveline, contain only arginine. Diprotamines, for example percine and cyprinine, contain arginine and either lysine or histidine. The triprotamines, such as sturine, contain all 3 amino acids. Bloch (1969,1976) separated the sperm basic proteins, according to cytochemical data, into 5 major classes. Type 1, or *Salmo* type, are easily extractable protamines whose sole basic amino acid is arginine. They are equivalent to the monoprotamines of Kossel (1928). They are also known as true or typical protamines. Type 2, mammalian type, are very basic and arginine rich, but they also have sulfur-containing amino acids and are only extractable after breaking disulphide linkages. These have also been called stable protamines or basic keratins. Type 3, *Mytilus* type, are easily extractable proteins that are intermediate in composition between the monoprotamines and somatic histones. They are the

di- and triprotamines designated by Kossel (1928). Type 4, *Rana* type, are sperm proteins which are apparently the same as the somatic histones. Type 5, crab type, have no basic proteins in the nucleus. Because some species may have sperm proteins which fall into more than one of the above categories, Subirana (1983) categorized them into 2 broad groups, protamines and histones. He defined the protamines as proteins with a lysine plus arginine content of 45 - 80% and a serine plus threonine content of 10 - 25%. This grouping combines the type 1, or "true", and type 2, or "stable", protamines and some of the type 3, or *Mytilus* type, intermediate proteins of Bloch (Oliva and Dixon, 1991). Four different nuclear compositions comprise Subirana's histone group: 1. no detectable change in the H1 family; 2. slight changes in the H1 family; 3. additional sperm-specific basic proteins; and 4. considerable changes in histones H1 and H2b (Subirana, 1983).

1.3 Properties

1.3.1 Typical Protamines

This section will focus on the arginine-rich protamines such as salmine and clupeine which, under the classification schemes given above, would be the type 1 protamines of Bloch (1969,1976) or the monoprotamines of Kossel (1928). These protamines were originally isolated from a number of fish species, particularly Salmonids and Clupeids, and have since been found in many other organisms including mollusks, amphibia, reptiles, birds, and metatherian mammals (Dixon *et al.*, 1985). Typical protamines have a number of interesting characteristics. One of the most striking is their highly basic nature.

Miyake (1927) determined the isoelectric point (pI) of a number of fish protamines and found that most had a pI of about 12. These proteins contain a high proportion of arginine residues -approximately two-thirds of all the amino acids in the polypeptide. In addition, all typical protamines contain alanine and serine, most contain proline and valine, many contain glycine and isoleucine and some contain threonine. Aromatic, and sulfur containing amino acids are not often present. Exceptions to this would be thynnine from *Thynnus thynnus* (tunny) and cyclopterine from *Cyclopterus lumpus* which contain tyrosine and mugiline β from *Mugii japonicus* which contains methionine (Ando *et al.*, 1973). Protamine from rooster sperm or Galline is also relatively high in tyrosine (Daly *et al.*, 1951). There is also a striking tendency for the basic and neutral amino acids to be clustered. For example, in the salmonid fishes, there are 4 clusters of 4 to 6 arginine residues present in a protamine molecule (Subirana, 1983). The actual peptide chain of typical protamines is short, often having less than 35 amino acids (Dixon *et al.*, 1985) and the molecular weight ranges from approximately 4000 to 5000 Da (Felix, 1960). Galline which has 65 residues, however, is about twice as long. This increased length seems to be the result of a partial gene duplication event that occurred during bird evolution (Dixon *et al.*, 1985). Despite the small size, these protamines are often heterogeneous and can be made up of several components which differ slightly in charge and length (Chevaillier, 1983). For example, clupeine can be fractionated into its three component proteins, namely clupeine Y-I, YII and Z, via ion exchange chromatography (Ando and Watanabe, 1969). The ultra-violet absorption

spectra of protamines generally have no maxima in the region above approximately 200 nm except for those containing aromatic amino acids. There is a peak present at about 200 nm which is ascribable to the absorption of peptide bonds. Since they have a relatively simple structure and are of low molecular weight, they do not denature or coagulate on heating (Ando *et al.*, 1973).

1.3.2 Stable Protamines

Stable protamines, or basic keratins are those referred to as type 2 protamines in the classification scheme of Bloch (1969,1976). They are found in all eutherian mammals, some insects, and an amphibian. Stable protamines are arginine-rich, like the typical protamines, however they also contain about 6-9 half-cystine residues which results in a great mechanical and chemical stability (Dixon *et al.*, 1985). The amino acid diversity, which is the number of different amino acids present in a protamine molecule, ranges from 8 - 13 (Bellvé, 1979). Tyrosine is present in almost all mammalian protamines (Chevaillier, 1983) and serine as well as threonine are also commonly found.

There are 2 classes of stable protamines, namely protamine 1 (or P1) and protamine 2 (or P2). While the sperm of every mammal examined so far contains protamine 1, only certain species such as mouse, hamster, human, and horse have been shown to contain protamine 2 (Pirhonen *et al.*, 1989). The protamine 2 gene is also present and transcribed at low levels in other mammals, however protamine 2 itself is not present in mature sperm (Maier *et al.*, 1990).

1.3.2.1 Protamine 1

These protamines typically contain about 50 residues with arginine comprising approximately half of them (Chevaillier, 1983). The molecule is made up of three domains: a central basic core and two less basic regions at the amino and carboxyl terminal ends (Hecht, 1989). The amino terminus is a hexapeptide sequence (alanine-arginine-tyrosine-arginine-cysteine-cysteine) which appears to be unique to protamine 1. The central region is arginine-rich, containing multiple arginine clusters separated by one or more other amino acids, and is believed to be the DNA-binding domain (Balhorn, 1989). The carboxyl terminal domain is highly conserved in length, but not in sequence (Hecht, 1989). Both the amino and carboxyl terminal domains contain serine, threonine, or tyrosine residues in specific locations which are probably phosphorylation sites (Balhorn, 1989).

1.3.2.2 Protamine 2

Protamine 2 is generally longer than P1 and is also more variable in length. The amino acid sequence of P2 does not appear to be as conserved between species. P2 also has a high content of histidine, an amino acid which is rare in P1 molecules. In protamine 2 the arginine clusters are spread rather evenly through the molecule rather than being concentrated in a central domain. The carboxyl terminal region is also variable, both in sequence and in length (Balhorn, 1989).

1.4 Electrospray Mass Spectrometry (ES-MS)

1.4.1 What is ES-MS?

A mass spectrometer performs three basic functions: produces gas-phase ions from sample molecules (accomplished in the ion source), separates gas-phase ions according to their mass-to-charge ratio (takes place in a mass analyzer), and detects and records the separated ions (Chapman, 1996). Electrospray (ES) is a relatively new ionization technique used in mass spectrometry which allows for rapid, accurate and sensitive analysis of a variety of molecules ranging in molecular weight from less than 200 Da. to greater than 100 KDa (Banoub *et al.*, 1995). In practice, it involves passing a sample solution through a hypodermic needle held at high voltage. The resulting high field at the tip of the needle disperses the sample as a fine spray of charged droplets which are driven by the field to the end plate of the source chamber a few centimeters away. The sign of the applied voltage determines the polarity of the droplets and ultimately the ions they will produce. A countercurrent flow of warm drying gas, usually dry nitrogen, helps to evaporate the solvent, and the charged droplets decrease in size until they become unstable and explode into a number of smaller droplets. When the droplets get small enough, ionized sample molecules will desorb under the influence of the field. These ions emerge into the first stage of the vacuum system and then pass through a skimmer into the second vacuum stage containing a mass analyzer. The resulting ES-MS spectrum is made up of a series of peaks of multiple charged ions (Chapman, 1996; Mann *et al.*, 1992). These raw data are usually processed via computer software to obtain a single mass peak representing the molecular weight of the compound.

1.4.2 ES-MS of Protamine

Since the 1990's, a few protamines have been analyzed via ES-MS and three such studies will be described here. Okamoto and colleagues (1992) used ES-MS to confirm the presence of two components in sardaine (Z1 and Z2), a protamine isolated from *Sarda orientalis*. The spectrum contained two series of multiple charged ions, A and B, with measured molecular masses of 4612.49 ± 0.74 Da and 4596.09 ± 1.10 Da. This corresponded to the predicted masses of sardaine Z1 and Z2 suggested from previous amino acid sequencing. Schindler and colleagues (1991) found that the ES-MS spectrum of protamine St1 from stallion had three series of peaks, A, B and C. The A series corresponded to the molecular mass of the expected protein (6998 Da.), while B and C were higher in molecular weight. The authors speculated that the 57 Da increase of mass in B was due to an amino acid substitution, an extra glycine, or an extra carboxamidomethylation. However, the authors offered no explanation for the 143 DA increase in mass for peak C, other than stating that the peak was a very minor unknown component that was probably structurally similar to A and B. The authors also analyzed the protamine Sp from cuttlefish in the same paper. The ES-MS spectrum showed 4 series of peaks, A, B, C and D with A and C representing the major peaks. Peak C was designated as protamine Sp1 and had a molecular mass of 8410 ± 1 Da, which was 397 Da heavier than the mass they had previously determined for Sp via amino acid analysis. This difference between the expected mass and the measured mass was thought to correspond to one serine and two arginine residues. Peak A was designated as protamine Sp2 and had a molecular mass of 8253 ± 1 Da, which was 240 Da

heavier than Sp. This was thought to correspond to one serine and one arginine residue. These speculations were confirmed in an accompanying paper (Martin-Ponthieu *et al.*, 1991). The other two series of peaks, B and D, represented minor compounds whose relationship to A and C was not determined (Schindler *et al.*, 1991). Kouach and colleagues (1993) found, by automated amino acid sequencing and mass spectrometry, that the originally published sequence of dogfish protamine Z3 (Sautiere *et al.*, 1981) was incorrect. The ES-MS spectrum showed two series of peaks, A and B, with molecular weights of 4748.8 ± 0.1 Da and 4846.6 ± 0.4 Da respectively. Series A was the major series of peaks and corresponded to the molecular weight of protamine Z3 determined by automated sequencing. This sequence was longer than the original one by a C-terminal hexapeptide. Series B was 98 Da heavier than A, and the authors attributed this to the presence of non-covalently bound adduct ions from sulfate or phosphate impurities in the sample.

1.5 Structure of Protamine and Nucleoprotamine

1.5.1 Protamine

Various data indicate that protamine, in most aqueous solutions, is in some extended or random coil form rather than an α-helical configuration (Ando *et al.*,1973). However it has also been documented that clupeines can partially adopt an α-helical form in structure-supporting organic solvents (Toniolo, 1980). Ottensmeyer and colleagues (1975) suggested that clupeine Y-I, and probably most other fish protamines, consist of a fairly loose, irregular coil

with the arginine side groups splayed away from each other in each loop or turn of the coil. They deduced this three-dimensional model using high resolution, dark-field electron microscopy, along with sequence and other data.

1.5.2 Nucleoprotamine

There have been a number of models proposed for the structure of nucleoprotamine. Feughelman and colleagues (1955) suggested a model, based on x-ray diffraction analyses, in which fully extended protamine winds around the DNA helix, with its arginine side-chains pointing out in opposite directions to interact with the phosphate groups of DNA. Luzzati and Nicolaieff (1963) proposed a second model which consists of hexagonally arranged DNA molecules with protamine and water filling up the gaps. Suau and Subirana (1977) suggested that protamines wrap around the minor groove of individual DNA molecules and that these DNA molecules pack in a hexagonal system. They also suggested that a small fraction of the protamines could cross-link different DNA molecules. Using data obtained from protamine-transfer RNA studies, Warrant and Kim (1978) proposed a model in which protamines, in the presence of nucleic acids, are composed of 3-4 α-helical domains that contain 4 or more consecutive arginine residues connected by 2-3 flexible joints. Each α-helical domain could lie along either groove of the DNA double helix, more likely the major groove, with 2 arginines bonding to 2 phosphates across the groove of that helix and the other arginines bonding to phosphates of neighbouring double helices. Bazett-Jones and Ottensmeyer (1979), using dark-field and bright-field

electron micrographs of re-constituted protamine-DNA, suggested a model in which DNA is wound around folded protamine molecules, one supercoil per protamine. The data favoured a continuous folding of DNA around protamine with a local unwinding of the DNA helix, thus resulting in the formation of a right-handed supercoil. Balhorn (1982) postulated a model in which the central polyarginine region of mammalian protamines, or the entire protamine molecule in fishes, adopts an extended conformation and binds to DNA in the minor groove in a manner similar to that described by Feughelman *et al.* (1955). The arginine side groups interact with the phosphates of DNA on its own and a neighbouring DNA strand, resulting in both charge neutralization and cross-linking of DNA molecules. The N-terminal and C-terminal tails do not bind along the groove of DNA, but participate in the formation of hydrogen, hydrophobic and disulfide bonds both within and between protamine molecules. Thus, each protamine in mammalian sperm is cross-linked to the next by disulfide bridges, locking the protamine around the DNA. Ebert and colleagues (1992) studied the conformation of fish protamines using reverse micelles, a system which mimiced the low hydration state of naturally occurring DNA-protamine complexes. Their results suggested that the C-terminal regions of the protamines attained an α-helical conformation in the complex with DNA as suggested by Warrant and Kim (1978). However, the other regions probably exist in an extended form like that suggested by Feughelman *et al.* (1955). Thus, they suggested that the actual mode of interaction between protamine and DNA might be a combination of both models. The α-helical portion of protamine could be bound to the major groove

and the extended portion could interact with neighbouring molecules and thus be responsible for intermolecular bridging. Alternatively, protamine binding could take place via the non-helical extended portion in the minor groove and the C-terminal α-helical portion could cause the intermolecular bridging.

1.6 Clinical Uses

Protamine is used today for two clinical purposes: the reversal of heparin-induced anticoagulation in patients undergoing certain procedures such as cardiac or vascular surgery, and dialysis; and as a complexing agent in subcutaneous insulin preparations, such as NPH (neutral protamine Hagedorn) and PZI (protamine zinc insulin), to delay the absorption of insulin and thus prolong its effects (Gottschlich *et al.*, 1988; Horrow, 1985).

The ability of protamine to inactivate anticoagulants was discovered by Chargaff and Olson (1937). Later, Jaques studied the practical use of protamine as a heparin antagonist for clinical purposes, developed a protamine titration assay and documented the *in vivo* neutralization of heparin using protamine (Jaques, 1973). Hagedorn and colleagues (1936) discovered that the effects of protamine insulinate, which is a mixture of protamine and insulin, were more prolonged than ordinary insulin thus diminishing blood sugar fluctuations and reducing subsequent ill effects.

Additional clinical uses for protamine were also investigated. A small number of studies in the 1960's reported that protamine inhibited the growth of cancerous tumours (O'Meara and O'Halloran, 1963; Hughes, 1964; Muggleton *et*

al., 1964; Lutton, 1964; Csaba *et al.*, 1960; Garvie, 1965). A more recent investigation suggested that protamine inhibits angiogenesis which is the formation of new blood vessels. Angiogenesis inhibitors would be useful in treating a number of diseases common in ophthalmology, dermatology, rheumatology and certain cancers. However, protamine was found to be toxic when administered systemically in doses large enough to reach the appropriate tissue concentration at the tumour site (Taylor and Folkman, 1982).

1.7 Antimicrobial Activity

1.7.1 Early Studies

McClean was the first to document the inhibition of microorganisms by protamine. Motivated by a desire to determine the substance in rabbit testicular extract which caused an enhanced activity of *Vaccinia* virus, he decided to test the effect of protamine which he knew was present in fish gonads. Contrary to his expectations, protamine inhibited the virus (McClean, 1930). He therefore decided to test the effect of protamine on bacteria and found that clupeine sulfate inhibited the growth of *B. typhosus* in broth. Also of interest was the fact that clupeine caused an immediate precipitate in the broth tubes containing higher concentrations of the protamine (McClean, 1931). Reiner and colleagues (1942) were the second group to report an inhibitory effect of protamine on microorganisms. While testing the toxicity of basic proteins used in insulin preparations, they found that protamine greatly inhibited the respiration of *Trypanosoma equiperdum*. Later that year, Miller and colleagues published the results of a study initially performed to determine if

protamine could be used to sensitize Gram-negative bacteria to antimicrobials that ordinarily acted only on Gram-positive organisms. They found that mixtures of salmine sulfate and any of the 3 antimicrobials tested, completely inhibited the respiration, as determined in phosphate buffer using Warburg respirometers, of *Escherichia coli*, whereas the antimicrobials alone had no effect. They discovered that salmine itself had an inhibitory effect and decided to test it against a number of bacteria. They found that it inhibited the respiration of a variety of Gram-positive and Gram-negative bacteria. They also tested the bactericidal activity of protamine and found that it paralleled the effect on bacterial metabolism (Miller *et al.*, 1942). About 14 years later, Kleczkowski and Kleczkowski (1956) studied the effect of clupeine on a strain of root nodule bacteria (*Rhizobium leguminosarum*), its bacteriophage, and the interaction between the two. In a nutrient broth used to grow root nodule bacteria, 0.02-0.05% clupeine killed the *Rhizobium* and slowly inactivated the virus. Further studies indicated that clupeine, in bactericidal concentrations, prevented the virus and bacteria from combining and also interrupted the normal course of phage development. They also tested the bactericidal activity of clupeine against *Escherichia coli* and *Staphylococcus aureus* and found that 0.05% clupeine killed all of the *E. coli* and most of the *S. aureus* when they were suspended in saline. However, when suspended in a nutrient broth typically used to culture these bacteria, clupeine produced a large precipitate and both organisms grew at their normal rates. Hirsch (1958) tested the activity of protamine on *Escherichia coli* K-12 and *Klebsiella pneumoniae* using a citrate-phosphate buffer system at pH 5.6 and 7.0. He used protamine concentrations up to 100

μg/mL and measured activity as the minimal concentration of protein which would produce >50% reduction in bacterial numbers in 2 h at 38°C. He found that for *E. coli*, at pH 7.0, the minimal concentration was 6 μg/mL and at pH 5.6 it was >100 μg/mL and stated similar results were obtained for *K. pneumoniae*. From this, he concluded that protamine had only a slight antibacterial activity at pH 7.0 and essentially no effect at pH 5.6. Brock (1958) wanted to examine the effects of salmine on bacteria in more detail. He chose 8 Gram-negative and 7 Gram-positive bacteria and grew each in tubes containing brain heart infusion broth and different concentrations of protamine. The lowest concentration of salmine, which gave complete absence of visible growth after incubation for 18 h, was designated as the minimum inhibitory concentration (MIC). The MIC's for the Gram-positive organisms ranged from 8 to >1000 μg/mL with all but 2 ≤ 125 μg/mL whereas for the Gram-negative species, the MIC's ranged from 32 to >1000 μg/mL with all but 2 strains ≥1000 μg/mL. Thus, under these conditions, salmine generally had a weaker antibacterial effect against Gram-negative organisms than against Gram-positive ones. Brock also tested the bactericidal, or killing effect, of salmine on 5 of the organisms. He included sensitive and more resistant strains from Gram-positive and negative species and found that salmine was bactericidal against all 5 organisms when suspended in water, but not when suspended in broth. Braekkan and Boge (1964) found that the growth of *Saccharomyces carlsbergensis* was stimulated in the presence of low concentrations of a raw extract of herring gonads whereas higher concentrations produced growth inhibition. They determined that the growth stimulatory factor

was vitamin B_6 and the inhibitory factor was protamine. They further purified this protamine (clupeine) and tested it against *Streptococcus faecalis*, 3 lactobacilli, and 3 fungal strains. Amounts of clupeine required for growth inhibition ranged from 5-10 μg/mL for the lactobacilli, 160 μg/mL for *S. faecalis* and 0.8-50 μg/mL for 2 of the fungi. Growth promotion was observed for the third fungal strain, *Aspergillus niger*. The authors suggested that this fungus may secrete enzymes which digest the protamine and the liberated amino acids result in growth stimulation. Using the experimental conditions of genetic transformation, Antohi and Popescu (1979) found that salmine exhibited a bactericidal effect on competent *Bacillus subtilis* cells. The number of surviving bacteria, determined by plate count, decreased significantly in protamine concentrations of 40 μg/mL and continued to decrease, with a survivability of less than 10% at protamine concentrations of 60-200 μg/mL. This represents most of the early information on the antimicrobial activity of protamines.

1.7.2 Recent Research

In the past 25 years, a number of detailed studies have been performed to examine the antimicrobial activity of protamines more closely. In particular, a Japanese research group led by Islam has published a series of articles on protamines. Islam and colleagues (1984) investigated the antibacterial activity of clupeine and salmine against a variety of Gram-positive and Gram-negative bacteria. First they screened 17 bacterial species using 500 μg/mL of protamine incorporated into agar plates. Of the 7 Gram-negative

organisms tested, only *Enterobacter aerogenes* showed any degree of growth inhibition. The rest were resistant at this concentration and were excluded from further study. Growth of all 10 of the Gram-positive organisms was inhibited. Next, the authors determined the minimum inhibitory concentration (MIC) for the susceptible species by inoculating a series of agar plates with different concentrations of protamine (agar dilution assay). The MIC was defined as the lowest concentration of protamine giving complete inhibition of bacterial growth after incubation for 20 h at 37°C. The MIC's, with clupeine, ranged from 75-400 μg/mL for the Gram-positive organisms and a value of 650 μg/mL was obtained for the Gram-negative bacterium, *Enterobacter aerogenes*. With salmine, the MIC's were either identical to, or within 50 μg/mL higher than, those obtained for clupeine. The authors also tested the lethal effect of clupeine on 6 *Bacillus* spp. using a broth assay and standard plate count. They found that the survivability of 5 of the 6 bacilli tested reached near zero at clupeine concentrations of 200-300 μg/mL. Only *B. lichenformis* was resistant to its lethal effect. In 1985, Islam and colleagues fractionated clupeine, into its three components Y-I, Y-II and Z, and salmine into two components S-A and S-B, and compared the antibacterial activity of the fractions with that of whole clupeine and salmine against the 6 *Bacillus* spp. used previously. In an agar dilution assay , MIC's for clupeine fractions Y-I and Y-II and salmine S-A were generally identical to or slightly higher than whole clupeine or salmine. For clupeine Z and salmine S-B, the MIC's were usually slightly lower than their unfractionated counterparts. The authors concluded that the difference in antibacterial activity of the fractionated

and whole protamines was not significant enough to warrant further study (Islam *et al.*, 1985b). Islam and colleagues (1986a) determined the effect of clupeine and salmine on the growth from spores of *Bacillus subtilis ruber* and *B. licheniformis* using both agar and broth dilution methods. The growth from spores of both organisms was inhibited by 100 -150 μg/mL protamine in the agar assay and by 10-50 μg/mL in the nutrient broth assay with the higher numbers in these ranges generally required for higher spore loads. Furthermore, they noted that the level of protamine required to prevent spore outgrowth was lower than the levels they had previously determined (Islam *et al.*, 1984) for growth inhibition of vegetative cells of these species. In an accompanying paper (Islam *et al.*,1986b) they found that heat treatment at 95°C combined with addition of protamine was effective in lowering the level of protamine or heating time necessary to prevent growth from spores of the *Bacillus* organisms tested. Kamal and colleagues (1986) tested the inhibitory effect of salmine on 15 strains of molds. The15 strains were first screened using 500 μg/mL salmine in an agar dilution assay. One strain, *Aspergillus niger*, showed growth promotion, 4 strains were resistant and 10 were sensitive to salmine at this concentration. The MIC for the 10 sensitive organisms ranged from 250-1000 μg/mL. In 1992, Yanagimoto and colleagues compared the antibacterial activities of salmine and browned salmine against 17 bacterial strains. Browned salmine was prepared by heating salmine with xylose at 100°C (pH 7.0) for 10 h. In an agar dilution method, the 5 Gram-negative organisms as well as 2 Gram-positive strains, were resistant to salmine and browned salmine at a concentration of 500 μg/mL. The

other Gram-positive organisms were sensitive with MIC values ranging from 150-525 μg/mL for salmine and 75-475 μg/mL for browned salmine. The MIC's for browned salmine were significantly lower than salmine in 3 species and only slightly lower in the other 7 organisms. Uyttendaele and Debevere (1994) tested the antibacterial activity of salmine against the foodborne pathogens *Bacillus cereus* and *Listeria monocytogenes* using the non-selective enrichment medium TSB (tryptic soy broth). Addition of salmine to TSB resulted in a precipitate, thus inhibition was measured by dilution and plate count. Salmine was bacteriostatic against *L. monocytogenes* at 1000, and 2500 ppm but had no effect against *B. cereus* at these concentrations. At 5000 ppm, salmine was bactericidal against both species. From a series of experiments they concluded that the antibacterial activity was a result of the soluble portion of protamine in broth. The pH and salt concentration also affected the activity with higher amounts of protamine needed at acidic pH or increased salt concentration. The MIC, defined as the lowest concentration of salmine which inhibited growth of the organism after incubation at 30°C for 24h, of these bacteria using an agar dilution assay was 700 ppm for *L. monocytogenes* and 1000 ppm for *B. cereus*. Johansen and colleagues (1995) tested the antibacterial activity of salmine against Gram-positive and Gram-negative bacteria using impedimetric measurements. With this method, the medium containing test bacteria and varying concentrations of salmine was monitored until a significant detectable increase in electrical conductivity was registered. This is referred to as the detection time (DT) and detection usually occurs when the bacterial cell concentration reaches 10^6-10^7 CFU/mL. The MIC

was determined as the lowest concentration of protamine resulting in an absence of DT after 100 h incubation. The MIC ranged from 20-1000 μg/mL for the 4 Gram-positive strains tested and from 500 to >4000 μg/mL for the 9 Gram-negatives. They also determined that 50-500 μg/mL protamine had a bactericidal effect on non-growing cells of *Shewanella putrefaciens* but had no such activity on *Listeria monocytogenes* even at 1000 μg/mL.

Thus, there is general agreement that protamines are effective against Gram-positive organisms. However, many authors have determined that the Gram-negative organisms are either much less sensitive or completely resistant to protamine at concentrations which inhibit the growth of Gram-positives. Protamine has also been shown to inhibit the growth from spores in a number of *Bacillus* species and to inhibit certain molds.

1.7.3 Factors Influencing Activity

A number of authors have observed, in the course of conducting their experiments, conditions which influenced the antimicrobial activity of protamines. Miller and colleagues (1942) noted that the presence of blood, serum, and broth caused a considerable reduction in antibacterial activity of salmine. They also stated that it had a greater activity at pH 8 or 9, than at pH 7, and was completely inactive at pH 5. Hirsch (1958) also found that protamine had slight antibacterial activity at pH 7 and essentially none at 5.6. Kleczkowski and Kleczkowski (1956) determined that clupeine had a greater activity against *Rhizobium* in broth media than in agar and, as stated previously, it had

bactericidal activity against *E. coli and S. aureus* in saline but not in nutrient broth. Similarly, Brock (1958) found that protamine had bactericidal activity against bacteria suspended in water but not in broth.

Because of a lack of specific information, detailed studies were carried out to test factors which could influence the activity of protamines. Islam and colleagues (1985a) studied the effects of pH, temperature, metal ions and organic matters on the bactericidal action of clupeine sulfate. The activity of 200 μg/mL clupeine was tested against *Bacillus* species using phosphate buffer as a basal medium, appropriately modified for different conditions. The bactericidal action was found to be higher at neutral and alkaline pH. The ionic strength of the solution greatly affected the bactericidal activity with a 62.5% bacterial survival rate in 0.5 M phosphate buffer as compared to a 0.14% survival rate in 0.01 M buffer. Temperature did not significantly affect the activity. Increased salt concentrations greatly inhibited the bactericidal activity with magnesium and calcium ions exerting a far more powerful effect than sodium or potassium ions. For example, the survival rate in 0.6 M NaCl was 31.4% whereas in 0.2 M $MgCl_2$ it was 84%. Organic substances, including 0.1 M glucose, 1% peptone, 0.5% meat extract, Penassay broth, tryptic soy broth and heart infusion broth, had very little or no effect on the bactericidal activity. This last finding contradicts those of the authors cited above, and Islam and colleagues suggest that this can be explained by differences in preparation of protamines, organisms involved and experimental conditions employed in different studies. Kamal and Motohiro (1986) performed a similar study to determine the factors that influenced the

effect of salmine on mold species. They too found that the inhibitory effect was greater at neutral or alkaline pH and that increased ionic strength or salt concentration increased survivability. Similarly Uyttendaele and Debevere (1994) determined that protamine had less activity against *Bacillus cereus* and *Listeria monocytogenes* at acidic pH or increased salt concentration. Islam and colleagues (1986a) found that the MIC of protamine, required to prevent outgrowth of *Bacillus* spores, was much lower in nutrient broth than agar. However, when enriched heart infusion broth or Penassay broth was used, the MIC's were generally much higher. The inhibitory effect of protamine was greater at pH 7 or 8 than at pH 6. In a companion paper, the authors tested the combined effect of heat treatment and clupeine on outgrowth from spores and found that in the nutritionally rich broth media, a higher level of protamine and longer heating time was required than in nutrient broth (Islam *et al.*, 1986b).

Thus, most authors agree that the type of medium, pH, and ionic strength or salt concentration have an influence on the antibacterial properties of protamines. The activity is generally higher in broth than in agar media, however in nutritionally rich broth there can be a decrease in its effects. The differences seen in agar and broth assays could be due to more protamine binding with constituents of the agar than the broth, or to a slower rate of diffusion through the agar (Kleczkowski and Kleczkowski, 1956). Diminished activity in nutritionally rich media may be because of binding of protamine with some organic matters and anionic substances (Islam et al 1986a). Protamine has a higher activity at neutral or alkaline pH. Islam and colleagues (1987) suggested that, at low pH, surface charges of the bacteria are altered which may

result in reduced binding and therefore the diminished activity of protamine. Aspedon and Groisman (1996) determined that protamine sensitivity is influenced by the energetic potential of the bacterial membrane and treatments that lower membrane potential, such as low pH, increase bacterial resistance. It is also more effective at low ionic strength or low salt concentration because salts are thought to compete with protamine for binding sites on the bacterial cell surface (Islam *et al.*, 1985a).

1.7.4 Mechanism of Action

The antibacterial activity of protamines is well established and it is assumed that this activity is a result of the polycationic nature of protamine because of its high arginine content. The actual mechanism involved is unknown, however a number of authors have suggested possible modes of action.

Brock 1958, observed that agglutination occurred in the presence of salmine with certain bacteria but determined that, in itself, this was not a sufficient explanation of bacteriostatic or bactericidal effects. He also noted that both Gram positive and negative organisms showed an initial marked increase in turbidity in the presence of salmine which peaked and then gradually returned to its original level. He postulated that this turbidity change was due to a direct effect of salmine on the cell surface resulting in an increase in the permeability of the cell to water. Protamine has also been shown to affect the permeability of artificially prepared cell membranes (Leitch and Tobias 1963, Larsen 1967). Antoji and Popescu (1979b) suggested that the primary site of action of

protamine was the cell wall. They found that salmine had lytic effects on strains of *Bacillus subtilis* but this occurred only under shaking conditions of incubation. This cell lysis, revealed by optical density measurements at 340 nm, started significantly at 100 μg/mL protamine. Bacterial lysis was apparently a multi-hit phenomen since it occurred only during incubation with shaking. Pretreatment of the protamine with digestive enzymes, or the addition of high molarities of salt or polyanionic lipopolysaccharide, inhibited bacteriolysis. These observations led the authors to suggest that protamine effects cell lysis by a multi-site wall component condensation, analogous to the mechanism of DNA compression in the sperm head. The model assumes that the polycationic protamine chains interact with polyanionic wall components causing extensive stabilizing of ionic bonds and inducing multizonal condensing areas. This results in wall splits and triggers cell lysis.

Because little information was available, Islam's group decided to perform a series of more detailed studies on the mode of action. Islam and colleagues (1987) suspended *Bacillus subtilis* and *B. licheniformis* in phosphate buffer and treated the suspensions with different concentrations of clupeine under static or shaking conditions. The treated cells were then appropriately prepared for scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM micrographs of protamine treated cells, using static incubation, showed surface depressions, decreased cell volume, surface granules, irregular contours, splits and blistering. Under shaking conditions, the micrographs showed that most of the cells were lysed. The TEM micrographs showed severe

wall damage, release of cytoplasmic materials, and in some cases lysis of the protamine treated cells. None of the above changes was observed in control (untreated) cells. Further experiments showed that protamine adsorbed to isolated cell walls and that this effect was greater at alkaline pH indicating that protamine had higher affinity for ionized sites of the cell wall. They also determined that there was increased leakage of cytoplasmic constituents from bacterial cells after treatment with protamine which indicated membrane damage. Thus the authors suggested that the interaction of the protamine polycation chain with polyanionic wall components might cause mechanical injury as well as damage to regulatory mechanisms that control cell permeability (Islam and collaegues,1987). In order to explain the membrane damage observed, Islam and colleagues (1987a) performed a study on the effects of clupeine on protoplasts of *B. subtilis*. They found that clupeine caused rapid lysis of protoplasts and the amount required for lysis was the same as that previously determined (Islam *et al.*, 1987) for the leakage of cytoplasmic materials from intact cells. Furthermore, isolated cell membranes or membrane components, particularly phospholipids, antagonized the antibacterial action of clupeine. They suggested that clupeine's strong affinity for phospholipids might be explained by electrostatic bonding of the ionic groups of the basic amino acids of clupeine with the phosphate group of the phospholipid. The authors concluded that this interaction might destroy the metabolic function of the membrane and lead to loss of cell viability. Islam and colleagues (1987b) carried out a further investigation to learn more about the cause of the loss of membrane function by studying the effect of clupeine on ATPase, an important

membrane-bound enzyme. Using isolated cell membranes of *B. subtilis*, they determined that clupeine caused a stimulation in ATPase activity. This stimulation could be blocked by the addition of phosphatidylethanolamine, a typical membrane phospholipid, suggesting that the added phospholipids interacted with the polycationic protamine, preventing it from reacting with anionic targets in the cell membrane. Thus it was determined that reaction with membrane phospholipids was required for protamine to act on the ATPase or the bacteria as a whole. The authors postulated that the stimulation in ATPase activity was caused by changes in the conformation of the enzyme resulting, either directly or indirectly, from the binding of clupeine with membrane phospholipids. From the results of the these three papers, the authors proposed the following hypothesis for the mechanism of action of clupeine against *B. subtilis*. Clupeine is at first taken up by the cell wall and then the cell membrane, with the latter being the main site of action. The bactericidal activity results from disruption of membrane structure because of an interaction with membrane phospholipid and a secondary destruction of membrane functions.

Other researchers also performed studies on the mode of action. Uyttendaele and Debevere (1994) determined from their experiments that the positively charged guanidine groups of the arginine-rich protamine were of great importance for the interaction with bacteria. They too, suggested that there is an electrostatic attraction between positively charged protamine and negatively charged cell wall polysaccharides or the phospholipid membrane, or both, which disturbs cell permeability. The severity of this disturbance would determine whether there was growth inhibitory, bacteriostatic, or bactericidal effects.

Furthermore they stated the inhibitory effect of salt on the antibacterial activity provided further evidence for the interaction of the polycationic protamine with the cell wall polysaccharides. The addition of NaCl would inhibit the effect of protamine, since the Na^+ ions would be attracted to the negative cell wall, and the Cl^- ions to the protamine. Aspedon and Groisman (1996) investigated the effects of protamine on the cytoplasmic membrane energization of *Salmonella typhimurium*. Experiments indicated that protamine sensitivity was influenced by the magnitude of the membrane potential and treatments that decreased membrane potential, such as low pH or the addition of respiratory poisons, increased bacterial resistance. The authors noted that growing cells were more susceptible to protamine than non-growing cells and determined, through experiments with cells that were energized but otherwise inhibited for growth, that an energized cytoplasmic membrane was required for antibacterial activity and not growth *per se*. Treatment with protamine also reduced the ATP content of the bacterial cells and inhibited amino acid transport, suggesting an impairment in the ability to generate a proton motive force. Thus, protamine inhibited several metabolic processes that rely on a functional cytoplasmic membrane without causing cell lysis or altering inner membrane permeability. The outer membrane permeability was disrupted by protamine, however the authors determined that this was not responsible for its antibacterial action.

1.8 Use as a Food Preservative

1.8.1 Background

In recent years, there has been increasing public interest and

concern over the use of chemical preservatives in foods. Protamine with its antibacterial and antifungal properties has drawn the attention of food additive users as a natural food preservative. Protamine has a number of other characteristics which make it an attractive choice as a food additive. It is reported that protamines are odourless, colorless, and almost tasteless (Islam et al, 1987a), they can be hydrolysed by digestive enzymes such as trypsin and chymotrypsin (Kleczkowski and Kleczkowski, 1956), and are heat stable (Brock, 1958). However, there are other factors which could limit its use. Because protamines exhibit greater activity at neutral or alkaline pH, they may not be as suitable for use in acidic food products. The composition of the food product is also important since the presence of organic matters and salts decreases the activity of protamine. Thus higher concentrations of protamine would be required to achieve the desired microbial inhibition. Another important factor is the possible toxicity of the protein. Studies have shown that injection of large doses of protamine are toxic to animals (Shelley *et al.*, 1942; Reiner *et al.*, 1942). There is also some controversy over possible allergic reactions to protamine in patients undergoing bypass surgery, who have had prior exposure to protamine in insulin therapy or who have had vasectomies (Pharo *et al.*, 1993; Watson *et al.*, 1983; Levy *et al.*, 1989; Gottschlich *et al.*, 1988).

1.8.2 Food Trials

Some research has been performed to test the activity of protamines in food. Yanagimoto and colleagues (1992) determined that browned salmine, at a level of 1 %, significantly lengthened the shelf-life of

Kamaboko (walleye pollack surimi). Matsudomi and colleagues (1994), with a view to improving the solubility and emulsifying properties of protamine in processed foods, tested the emulsifying and bactericidal properties of salmine conjugated to galactomannan which is a mannase hydrolysate of guar gum. They found that the conjugate had excellent emulsifying properties in acidic, neutral, and high salt (0.2 M NaCl) buffer systems and even outperformed the commercial emulsifiers tested. The conjugate retained its properties even after heat treatment at 90°C for 5 min. The bactericidal activity of the conjugate against *Escherichia coli* and *Bacillus cereus* was slightly lower than that of the unconjugated protamine-galactomannan mixture, however, the difference was not significant.

1.9 Objectives

Because much of the early research involving isolation of protamines was performed in Europe, my first goal was to isolate, purify, and partially characterize protamines of the herring (*Clupea harengus*) obtained off the coast of Newfoundland. The second objective was to compare the characteristics of these protamines with those available commercially. In light of the recent interest in protamine as a food preservative and the differing opinions on its effectiveness against Gram-negative bacteria, the third objective was to test the antibacterial activity of both isolated and commercial protamines against a variety of bacteria, including food-spoilage, foodborne pathogens and fish pathogens, with emphasis on Gram-negative organisms.

2. MATERIALS AND METHODS

2.1 Materials

The following chemicals and reagents were obtained from Sigma Chemical Company (St. Louis, Missouri, USA): sodium chloride, sodium ethylenediaminetetraacetic acid (NaEDTA), phenylmethylsulfonlyfluoride (PMSF), tris(hyroxymethyl)aminomethane (Tris), Folin and Ciocalteau's Phenol reagent, sodium potassium tartrate, sodium carbonate (Na_2CO_3), acrylamide, N',N'-methylene-bis-acrylamide (Bis), sodium dodecyl sulfate (SDS), N',N',N',N'-tetramethylethylenediamine (TEMED), tricine, bromophenol blue, ammonium persulfate, glycerol, coomassie brilliant blue R-250, 2-β mercaptoethanol, urea, β-alanine, potassium hydroxide, riboflavin, CM-Sephadex C-25, sodium acetate trihydrate, bovine serum albumin (BSA), protamine sulfate from herring (product # P4505) and protamine sulfate from salmon (product # P4005). Sulfuric acid (H_2SO_4), sodium hydroxide (NaOH) and glacial acetic acid were obtained from Fisher Scientific Company (Nepean, Ontario, Can.) and methanol from Fisher Scientific (Fair Lawn, New Jersey, USA). Copper sulfate pentahydrate ($CuSO_4 \bullet 5H_2O$) and amidoblack 10B were obtained from J.T. Baker Chemical Company (Phillipsburg, New Jersey). Mueller-Hinton broth (MHB) and Trypticase soy agar (TSA) were obtained from VWR Scientific (Mississauga, Ontario).

2.2 Samples

Extracts containing protamines were made from testes, stored frozen, of

herring, *Clupea harengus*, obtained from the Department of Fisheries and Oceans, St. John's, Newfoundland. The first batch of gonads obtained was from mature fish, but their reproductive stage was uncertain. The second batch was obtained from mature fish with ripe gonads. Commercial protamine (clupeine) from Sigma was used for comparison.

2.3 Protein Extraction

Nuclear protein was extracted from the gonadal cells using the method of Kennedy and Davies (1979). This procedure involved two main steps: obtaining the nuclei from the gonadal cells and subsequently extracting the acid soluble nuclear material.

To obtain the nuclei, 80-100g of partially thawed testes, from a number of fish, was weighed, scissor minced, and homogenized in a Polytron at medium speed, 4 periods, 30 seconds each in 3-4 volumes of 75mM NaCl containing 25mM NaEDTA, pH 8.0 and 0.5mM PMSF. The mixture was filtered through 4 layers of cheesecloth and the filtrate centrifuged at 1500 x *g* for 10 min. at 4°C using a Sorvall RC5C plus refrigerated centrifuge (DuPont, Newtown, Connecticut). The cellular material was re-suspended in the same buffer, centrifuged at 3000 x *g* for 10 min. at 4°C and again re-suspended in buffer.

The acid-soluble nuclear protein was extracted from the re-suspended cellular material by the addition of 10-15 volumes of 0.2M H_2SO_4 for 30 min. The material was centrifuged at 17000 x *g* for 20 min. at 4°C and the supernatant stored. The solid residue was subjected to a second extraction and centrifuged

as above. Protein was precipitated from the combined supernatants by the addition of 3-4 volumes of 95% ethanol and storage overnight at -20°C. The material was centrifuged at 13500 x *g* for 30 min. at 4°C and the protein dissolved in water and neutralized with 1M Tris base. The protein was re-precipitated with 3-4 volumes of 95% ethanol as above. The final precipitate was dissolved in water and stored at -20°C.

2.4 Protein Characterization

2.4.1 Determination of Protein Content

The Lowry method (Lowry *et al.*, 1951) was used to estimate the amount of protein present in the extract. A series of standards containing from 0-100 µg of protein in 0.2 mL were prepared in duplicate 12x75 mm or 13X100 mm disposable culture tubes using a 1 mg/mL solution of BSA. Aliquots of appropriately diluted sample protein were pipetted into separate tubes, in duplicate, and brought to a volume of 0.2 mL with distilled water. Then 1.0 mL of copper reagent was added to each tube, both standards and samples, mixed, and incubated at room temperature (RT) for 10 min. Then 0.1 mL of phenol reagent was added to each tube, mixed, and incubated at RT for 30 min (see appendix, section B). The absorbance was then measured, at 600 nm, using a Shimadzu Model UV-260 Recording Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). This spectrophotometer has a program which automatically produces a standard curve from the protein standards measured and then calculates the amount of protein present in the samples. The absorbance values

were also manually entered into sigma plot and a regression performed since the graph generated in sigma plot is more visually pleasing than that printed by the spectrophotometer.

2.4.2 Absorption Spectrum

Absorption spectra were performed in order to ascertain whether the extracts did indeed contain protamines and to check for the presence of contaminating gonadal proteins. Samples (1mL) of protein extract and commercial clupeine were centrifuged for 1 min in a Brinkman Eppendorf Model 5414 microcentrifuge (Brinkman Instruments, Rexdale, Ontario). Then, 40 μL aliquots were diluted to 1 mL with distilled water and the absorbance measured from 190-600 nm using the Shimadzu spectrophotometer.

2.4.3 Gel Electrophoresis

2.4.3.1 SDS-PAGE

SDS-PAGE was performed according to the method of Schagger and von Jagow (1987) as described in Sigma technical bulletin No. MWM-100 (1992). This method was designed to give a more accurate estimation of the molecular weight of small proteins which often give anomalous results using typical SDS-PAGE procedures. Separating, spacer, and stacking gel solutions were prepared as described in Table A.5 of the appendix. The separating gel was then poured between the glass plates to a height of 10 cm and the spacer gel overlaid another 2-3 cm. These were then overlaid with water

and left to polymerize. After polymerization was complete, the water overlay was removed. Then, a well-forming comb was put in place, and the stacking gel poured onto the spacer gel. The gels were left to equilibrate overnight. The next day, the wells were rinsed with water, followed by cathode buffer, Tris-tricine pH 8.2, and the gel assembly fitted to a HSI SE 600 vertical slab gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, California). Cathode buffer was poured into the upper buffer chamber and anode buffer into the lower buffer chamber. Samples, 10 to 20 μL, were diluted in 40 μL of sample buffer (see appendix), and heated at 40°C for 30 min. Molecular weight markers (Sigma- MW-SDS-17s) were reconstituted in 2.2 mL of sample buffer and 15 μl aliquots were heated along with the study samples for each gel run. The samples and markers were loaded into the wells, and electrophoresed at 21-60 mA until the tracking dye was within about 1 cm of the anodic end of the gel, approximately 6 hours. The gel was removed from the apparatus and the position of the tracking dye marked. Next, the gel was placed in fixative, 50% methanol / 10% acetic acid, for 1 h and 15 min., then transferred to stain, 0.025% Coomassie brilliant blue R250 in 10% acetic acid, for 2 h, and finally de-stain solution, 10% acetic acid, overnight with shaking. The positions of the bands were measured and relative mobility (R_f) values calculated. Gels were then stored in 10% acetic acid at 4°C.

2.4.3.2 PAGE

Polyacrylamide gel electrophoresis was done by the method

of Reisfeld *et al.*, (1962) but with the addition of 6-7M urea. PAGE was performed in an attempt to determine the number of component protamines in the extracts. Stock, buffer, and sample solutions and the formulation of the small pore and large pore gels are given in section C of the appendix. The small pore solution was prepared, degassed, and 12 mL of freshly prepared ammonium persulfate solution was added. The method originally required 24 mL of ammonium persulfate solution, however this amount caused the gels to become brittle and crack. Further experiments determined that 12 mL was sufficient to polymerize the gels and an extra 12 mL of water was added to the small pore gel solution to maintain the proper volume. Then 1.7 mL of the completed gel solution was pipetted into 7x125 mm tubes and overlaid with 0.1 mL of distilled water and allowed to polymerize for 30 minutes after which the water layer was removed. Meanwhile, the large pore solution was prepared, degassed and 1.875 mL riboflavin solution added. Then, 0.3 mL of this large pore gel solution was pipetted into the tubes, overlaid with 0.1 mL of distilled water and allowed to polymerize in daylight until the gel became opalescent, about 30 minutes to1 hour. The amount of riboflavin had to be increased from the required 1.5 mL to 1.875 mL to effect photopolymerization within the expected time frame. Next the water layer was removed and the tube gels attached to the Biorad 150A (Biorad Laboratories) vertical tube gel electrophoresis apparatus. β-alanine buffer, pH 4.5, was added to the upper and lower buffer chambers and 0.2 mL of sample solution was applied to each tube under buffer. Electrophoresis was carried out at 5 mA per tube for approximately 3 hours. Afterward, gels were removed from

the tubes by first rimming the tubes with a 21-gauge syringe needle through which distilled water was passed, followed by ejection of the gel with a pipette bulb. The position of the tracking dye, pyronine Y, was then marked with small pieces of wire. Gels were placed in stain, 1% amidoblack in 7% acetic acid, for 1 hr then de-stained in 7% acetic acid in a diffusion de-stainer until all the background dye was removed, about 1-2 days. The gels were then stored in screw-cap test tubes containing 7% acetic acid.

2.4.4 Ion Exchange Chromatography

The purpose of this procedure was to separate the component protamines in the extracts into separate fractions.

2.4.4.1 Preparation of the Column

CM-Sephadex C-25 (30 mg) was weighed on a top loading balance and then swollen in excess 1M NaCl for 5 min. The NaCl solution was removed and the beads were then swollen in excess equilibration buffer, 0.05M acetate buffer containing 0.5M NaCl, pH 5.8 (see appendix, section D), for 1-2 days. The gel was autoclaved at 121°C, 15 psi, for 15 min. to remove air bubbles and ensure sterility. After cooling, the gel was packed into a 1.5 x 100 cm column that had been pre-coated with a 0.5% solution of Photo Flo 200 (Kodak, Toronto, Ontario). The gel bed was stabilized by running 3-4 bed volumes of equilibration buffer through it.

2.4.4.2 Preparation of the Sample

The first crude protamine sample, combined extracts 1 and 2, was dialysed against equilibration buffer using Spectrapor 6 dialysis tubing, mwco 1000 (Spectrum Medical Industries Inc.,California USA). The tubing was prepared according to the procedure given in Maniatis *et al.*, (1982). First, a 15 cm length of tubing was boiled for 10 min. in approximately 1 L of 2% sodium bicarbonate and 1 mM EDTA. Next, it was rinsed in distilled water and then boiled for 10 min. in distilled water. After cooling, the tubing was rinsed inside and out with distilled water. A 10 mL sample containing approximately 75 mg of protamine was placed in the dialysis tubing, immersed in 800 mL of equilibration buffer, placed on a magnetic stirrer, and the buffer changed every 10-12 hours for 2 days. Lowry protein determination showed a reduction in protein from 75 to approximately 30 mg after dialysis. Thus, for comparison, 30 mg of commercial protamine (clupeine) was simply dissolved in the buffer, dialysis was unnecessary. Due to losses during dialysis, subsequent protamine samples were placed in an equal volume of double strength equilibration buffer before loading onto the column.

2.4.4.3 Column Chromatography

The equilibration buffer was removed from the top of the bed and the protamine sample loaded at the top of the gel. After it had completely migrated into the gel, the walls of the column were rinsed twice with equilibration buffer, the column filled with this buffer and attached to the buffer reservoir. The outlet of the column was attached to a fraction collector 2112

Redirac (LKB Bromma, Sweden). The flow rate was approximately 9-11 mL/h, and fractions of 3 - 3.5 mL were collected in 16X100 mm disposable culture tubes. After passing approximately 200 mL of the equilibration buffer through the column, the bound protein was eluted from the gel with 0.05M acetate buffer containing 1.5M NaCl. The protein in the fractions was monitored at 220 nm using the Shimadzu spectrophotometer until no protein was detected in the fractions collected. Fractions were pooled and the protein content determined. For protamine oil, the 6 fractions with the highest absorbance in each peak were pooled separately in order to obtain a purer sample. The column was washed between runs with 2 bed volumes of 0.1M NaOH and then re-equlibrated with the equilibration buffer as suggested by manufacturer.

2.4.5 Amino Acid Composition

Amino acid composition was performed by Sonya Banfield and Chris Skinner at the amino acid analysis faciltiy, MUN. Protamine samples (1 mL) were placed in 16X100 mm culture tubes with teflon lined screw caps and hydrolyzed *in vacuo* at 110°C for 24h in 1 mL of 6N HCl with 0.05% phenol. The HCl was removed under vacuum and the dried samples reconstituted with pH 2.2 sodium citrate buffer 0.2M Na^+ prior to analysis on a Beckman model 121 MB amino acid analyzer. No corrections were made for losses during hydrolysis. The amino acid compositions were calculated relative to the number of alanine residues.

2.4.6 Mass Spectra

Electrospray mass spectrometry was performed by Dr. J. Banoub and Steve Combden, Department of Fisheries and Oceans, St. John's, NF. This new and sensitive technique was used to more accurately determine the molecular weights of the protamines. Mass spectra of the fractionated components of clupeine (Sigma) and protamine oil were recorded, in positive ion mode, using a Micromass Quattro triple quadrupole mass spectrometer equipped with an electrospray ionization source capable of analyzing ions up to *m/z* (mass over charge) 4000. A pentium 166 personal computer equipped with Micromass MASSLYNX 2.22 Mass Spectrometry Data Handling System Software was used for data acquisition and processing. The temperature of the ES ionization source was maintained at 80°C. The operating voltage of the ES capillary was 3.50 kV, the cone voltage was set at 30 V, skimmer offset of 5 V and the high voltage lens was maintained at 0.50 kV. The mass scale was calibrated in the positive ion mode using a polyethylene glycol mixture. The ES spectra obtained were background-subtracted, smoothed, and centered. Maximum Entropy (Micromass Instuments), a mathematical technique based on rigorous probability theory, was used to reconstuct the underlying distribution of a spectrum into a single peak and then determine its molecular mass. The computer also generated a mock spectrum, based on predicted fragmentation patterns of molecules of each molecular mass, for comparison with the actual spectrum obtained.

2.5 Determination of Antimicrobial Activity

2.5.1 Test Organisms

A panel of food spoilage, food-borne pathogens, and fish pathogens was chosen as organisms to be used to investigate the antimicrobial properties of the extracted protamines. The following were obtained from our university stock collections of ATCC cultures: *Bacillus subtilis* #6051, *Escherichia coli* #11775, *Staphylococcus aureus* #12600 , *Salmonella typhimurium* #23564, *Pseudomonas fluorescens* #13525, and *Pseudomonas aeruginosa* #27853. *Listeria monocytogenes* serotype 4b is an isolate of human origin that was originally obtained from the LCDC, Ottawa, Canada. *Aeromonas sobria*, *Vibrio anguillarum*, *Aeromonas salmonicida*, and *Yersinia ruckeri* were obtained from the Marine Institute, MUN. These four were maintained on TSA plates at room temperature, sealed with parafilm to prevent drying and subcultured unto fresh TSA plates for testing. Backup cultures were kept on TSA slants at 5°C. All other cultures were maintained on TSA slants at 5°C and subcultured onto TSA plates for testing.

2.5.2 Broth Dilution Protocol

A series of 16x125mm test tubes were prepared containing different concentrations of protamine extract 3, 'oil' fraction, or commercial protamine in MHB pH 7.2 - 7.4 for most organisms. MHB was supplemented with 100mM NaCl for the second trial of *Vibrio anguillarum* and LEB was used for the assays with *Listeria monocytogenes*. Protamine concentrations used in each

assay ranged from 0-400 μg/mL for sensitive organisms to 0-1400 μg/mL for the most resistant organisms. For details of tube preparation see Table A.8 in appendix. To these tubes, 0.1 mL of a standardized inoculum was added. This standardized inoculum was prepared by inoculating isolated colonies from an overnight culture plate of the test organism into MHB. The turbidity of this inoculum was then adjusted with MHB to give an absorbance value of 0.08 - 0.10 at 625 nm on the spectrophotometer. According to the National Committee for Clinical Laboratory Standards (NCCLS), this OD range corresponds to approximately 1-2x10^8 CFU/mL (NCCLS, 1993). The adjusted inoculum was diluted 1:10 in MHB, so that, after addition of 0.1 mL to each tube, the resulting inoculum density would be approximately 4x10^5 CFU/mL. This density was checked by dilution and spread-plating of a sample removed from the growth control tube, lacking protamine, immediately after inoculation. The tubes and plates were then incubated under conditions appropriate for each organism: *Pseudomonas fluorescens*, *Aeromonas sobria*, *A. salmonicida*, *Vibrio anguillarum*, and *Yersinia ruckerii* , 24 - 48 h at room temperature; *Bacillus subtilis*, 30°C for 22 - 24 h; and *P. aeruginosa*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Staphylococcus aureus*, 37°C for 20 -24 h. After incubation, optical density measurements using the Shimadzu or Spectronic 601 (Milton Roy Co., USA) spectrophotometers were used to determine the degree of growth inhibition. These broth assays were performed twice using duplicate tubes for each trial. In the second trial, aerobic plate counts of the bacteria were performed in order to determine the bactericidal activity of the protamines.

3. RESULTS

3.1 Protein Content

Approximately 413 mg of protamine was obtained from the first batch of approximately 200 g of gonadal material. This represents the pooled amount of protamine from two separate protein extractions as determined by the Lowry method (Lowry *et al.*, 1951). The protein concentration was approximately 7.5 mg/mL and 55 mL were obtained. The raw data used to construct the standard curve (see Figure 1) and the calculation of the protamine concentration are given in the appendix (see Tables A.1 and A.2).

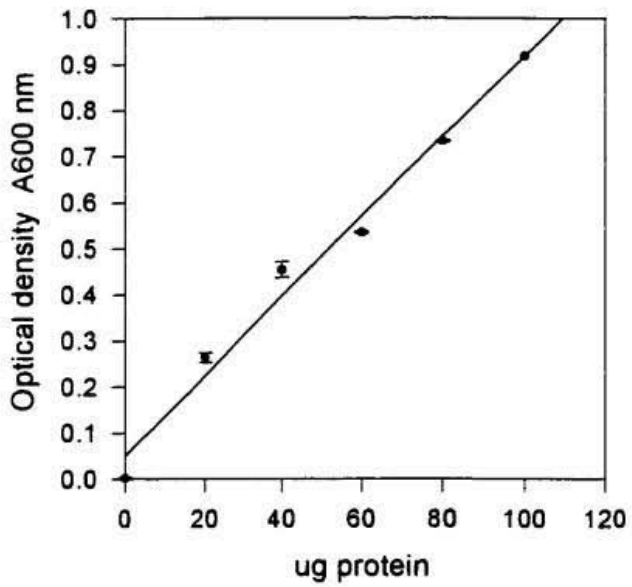


Figure 1: Standard curve for Lowry protein determination of combined protamine extracts 1 and 2.

The third protein extraction using 80 g of ripe gonads from the second batch of fish, yielded approximately 450 mg of protamine. Most of the protamine deposited as an oily substance which when removed and dissolved in 80 mL of water had a protein concentration of approximately 5 mg/mL. The non-oily, fraction, about 20 mL, had a protein concentration of approximately 2.5 mg/mL. The raw data used to construct this standard curve (see Figure 2) and the calculation of the protamine concentrations are given in Tables A.3 and A.4 of the appendix.

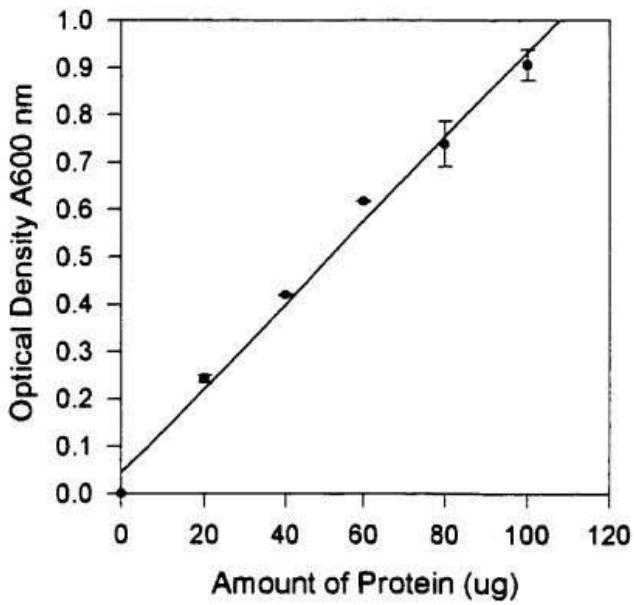


Figure 2: Standard curve for Lowry protein determination of third protamine extract.

3.2 Absorption Spectra

The absorption spectra for the isolated protamines from different batches of gonads and the commercial protamine are quite similar. All have only one major peak and it occurs in the wavelength range of 190-230 nm (see Figure 3).

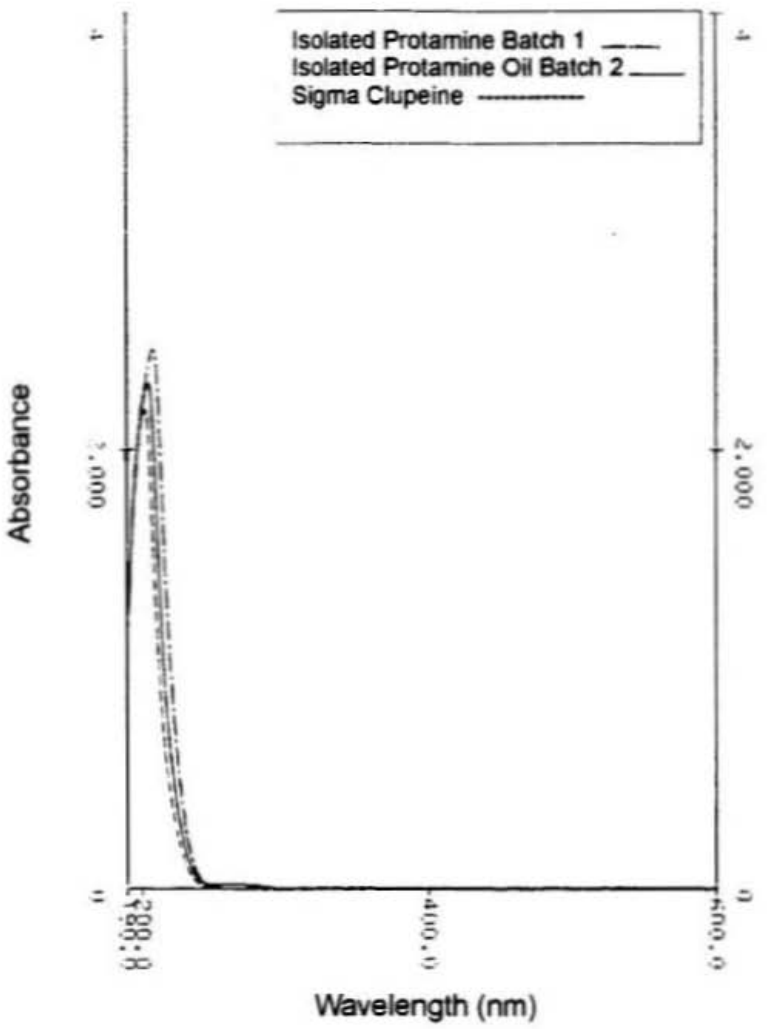


Figure 3: Absorption spectra of isolated protamines and Sigma clupeine

3.3 Gel Electrophoresis

3.3.1 SDS-PAGE

In lane 1, molecular weight markers, there are 6 bands observable in the photograph (see Figure 4). There were 7 bands present but the last one toward the bottom of the gel was rather faint and was lost during gel storage. For my latest protamine extract, oily fraction, there is one band (see lanes 2 and 6). For the protamine isolated from the first batch, first and second extracts combined, there are apparently 4 bands; a triplet and 1 broad band (see lane 3). There was no observable band for Sigma clupeine (see lane 4), however for salmon protamine, or salmine, from Sigma there is apparently 1 very broad band (see lane 5). From the R_f values determined for the 7 bands of the molecular weight markers, a standard curve was constructed to estimate the molecular weights of the protamines (see Figure 5).

The calculated R_f value for the band obtained for the oily fraction was 0.88 corresponding to an approximate molecular weight of 7100. For the extract from the first batch of gonads, the R_f values for the bands were 0.81, 0.82, 0.86, and0.92 corresponding to 9200, 9000, 7600, and 6200 respectively. Finally for salmine (Sigma), the R_f values calculated for the top, middle and bottom of the broad band were 0.84, 0.89 and 0.93 corresponding to molecular weights of 8200, 6800 and 5400 respectively.

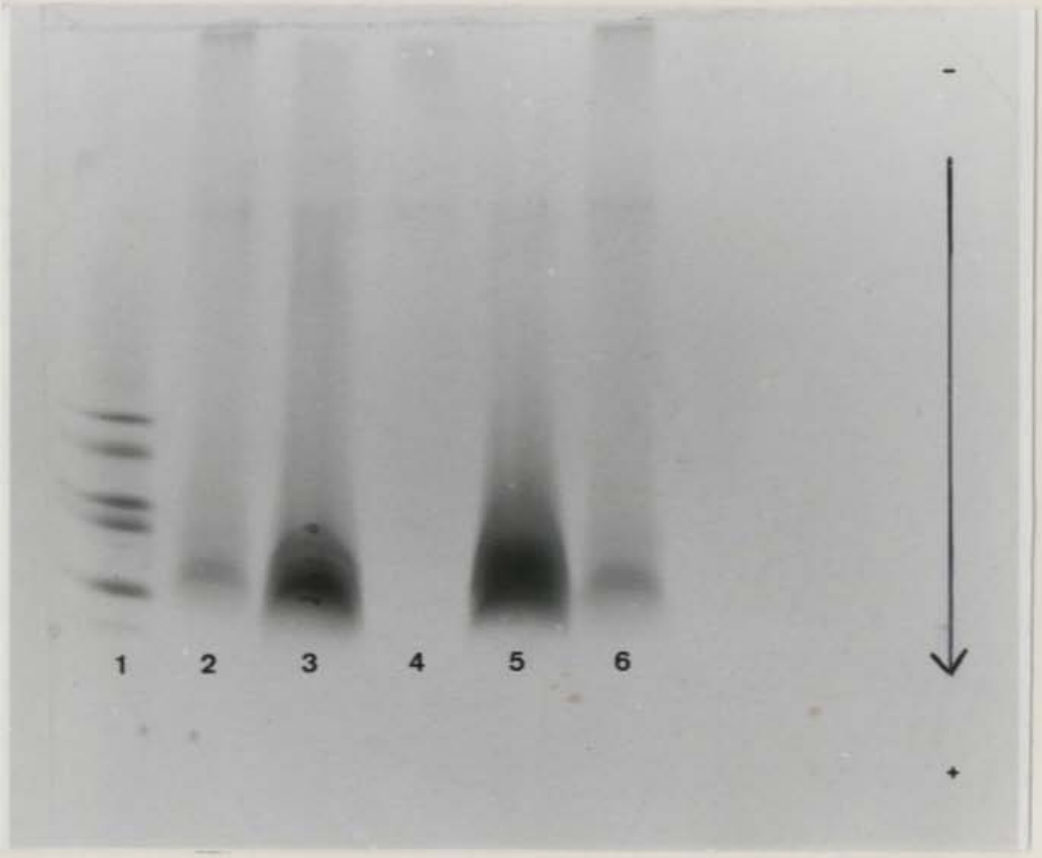


Figure 4: SDS-PAGE of protamines isolated from herring gonads and commercial protamines from Sigma.

The gel was run at 21 mA until the samples completely entered the stacking gel, 30 min. The current was then increased to 33 mA for 3 hr and then 60 mA for the final 2.5 hr. The gel was fixed in 50% methanol/10% acetic acid, and stained in 0.025% Coomassie brilliant blue R-250 in 10% acetic acid. Migration was from top (cathode) to bottom. Lane 1, molecular weight markers, 10 μg (Sigma MW-SDS-17s); lanes 2 and 6, isolated protamine oil fraction, 50 and 100 μg respectively; lane 3, protamine isolated from first batch of gonads, 75 μg; lane 4, clupeine (Sigma), 75 μg; lane 5, salmine (Sigma), 75 μg.

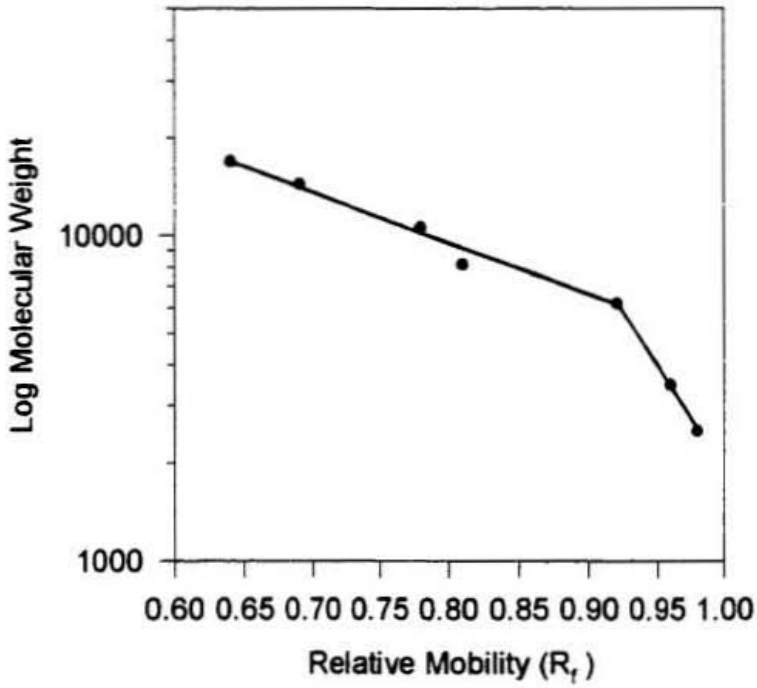


Figure 5: Calibration curve obtained after electrophoresis of the peptides from the MW-SDS-17S kit (Sigma).

3.3.2 PAGE

The results of polyacrylamide gel electrophoresis are given in Figures 6-8. The metal pins represent the final position of the tracking dye, pyronine Y. The bands are protamines stained with amidoblack 10B. With the exception of gels 1 and 2 in Figure 7 where there were no observable bands, every other tube gel had only one band (Figures 6 - 8). Gel 3 on Figure 7 did have a faint band but it was lost during gel storage.

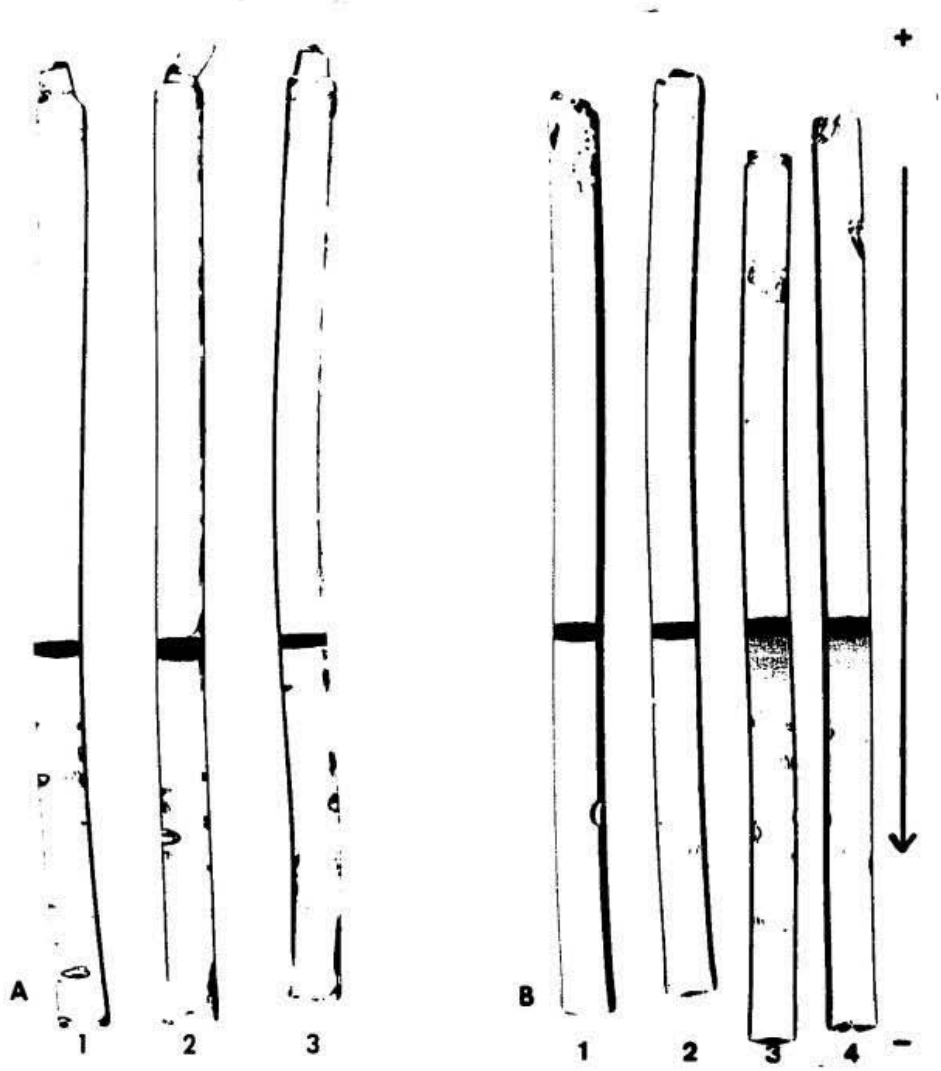


Figure 6: Polyacrylamide gel electrophoresis of protamines isolated from herring gonads and commercial protamines from Sigma.

Gels were run at a constant current of 5 mA per tube for 3 h and then stained in 1% amidoblack 10B in 7% acetic acid. Migration was from top (anode) to bottom. The stained bands are protamines and the wire pieces mark the position of the tracking dye, pyronine Y. (A) Crude extracts. Gel 1, protamine oil extract, 25 μg; gel 2, protamine from first batch of gonads, 37.5 μg; Gel 3, clupeine (Sigma), 25 μg. (B) Fractions obtained from ion exchange chromatography of protamine oil. Gel 1, fraction A, 6 μg; gel 2, fraction B, 4.5 μg; gel 3, fraction B *, 6 μg; gel 4, fraction C, 4.5 μg. (*sample taken from the entire pooled B peak)

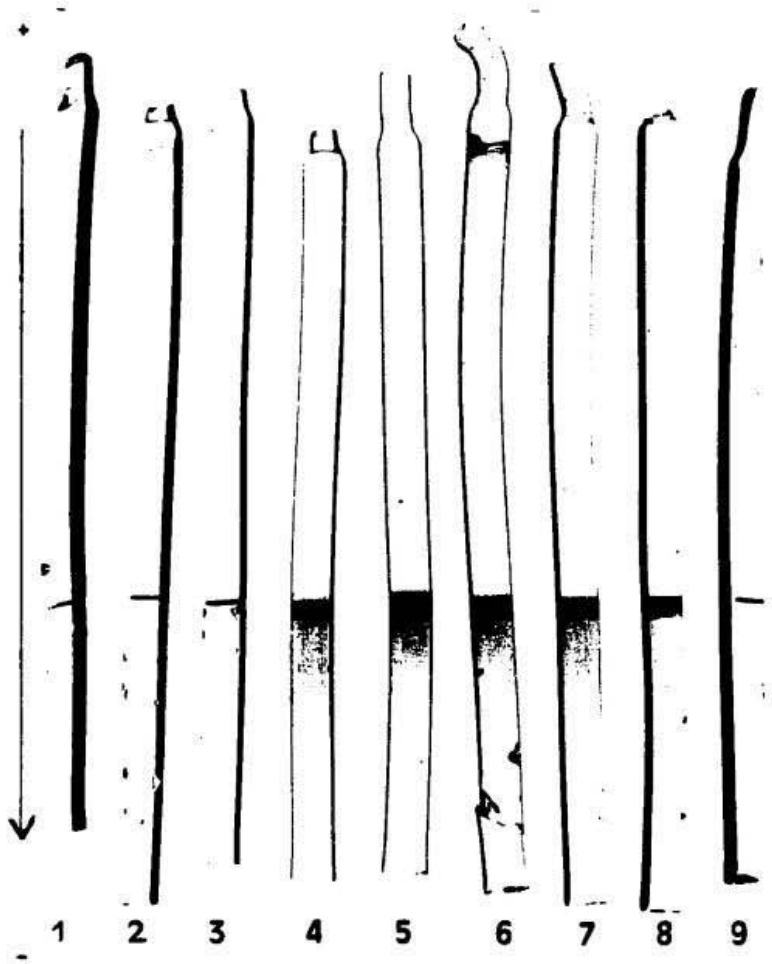


Figure 7: Polyacrylamide gel electrophoresis of fractions obtained from the ion exchange chromatography of the first batch of isolated protamines. Gels were run at a constant current of 5 mA per tube for 3 h and then stained in 1% amidoblack 10B in 7% acetic acid. Migration was from top (anode) to bottom. The stained bands are protamines and the wire pieces mark the position of the tracking dye, pyronine Y. Gel 1, fraction A, 60 µL; gel 2, fraction B, 3 µg; gel 3, fraction C, 6 µg; gel 4, fraction D, 6.5 µg; gel 5, fraction E, 5.5 µg; gel 6, fraction F, 6 µg; gel 7, fraction G, 6 µg; gel 8, fraction H, 5.5 µg; gel 9, fraction I, 2.5 µg.

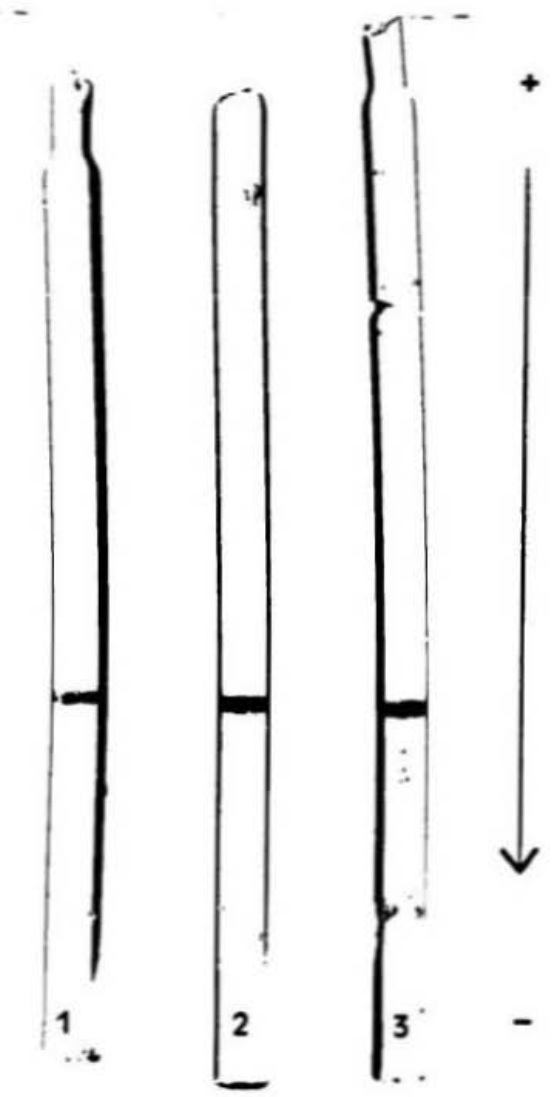


Figure 8: Polyacrylamide gel electrophoresis of fractions obtained from the ion exchange chromatography of Sigma clupeine.
Gels were run at a constant current of 5 mA per tube for 3 h and then stained in 1% amidoblack 10B in 7% acetic acid. Migration was from top (anode) to bottom. The stained bands are protamines and the wire pieces mark the position of the tracking dye, pyronine Y. Gel 1, fraction A, 3.5 μg; gel 2, fraction B, 3.5 μg; gel 3, fraction C, 3 μg.

3.4 Ion Exchange Chromatography

The results of the ion exchange chromatography of protamine from the first batch of gonadal material are shown in Figure 9. There is one peak very early in the procedure and a series of 8 peaks after the switch to elution buffer (arrow). The chromatogram for Sigma clupeine shows 3 large peaks (see Figure 10), all of which occur after switching to elution buffer. For protamine oil obtained from the third extraction (see Figure 11), there is one very early peak and, after switching to elution buffer, 3 more large peaks.

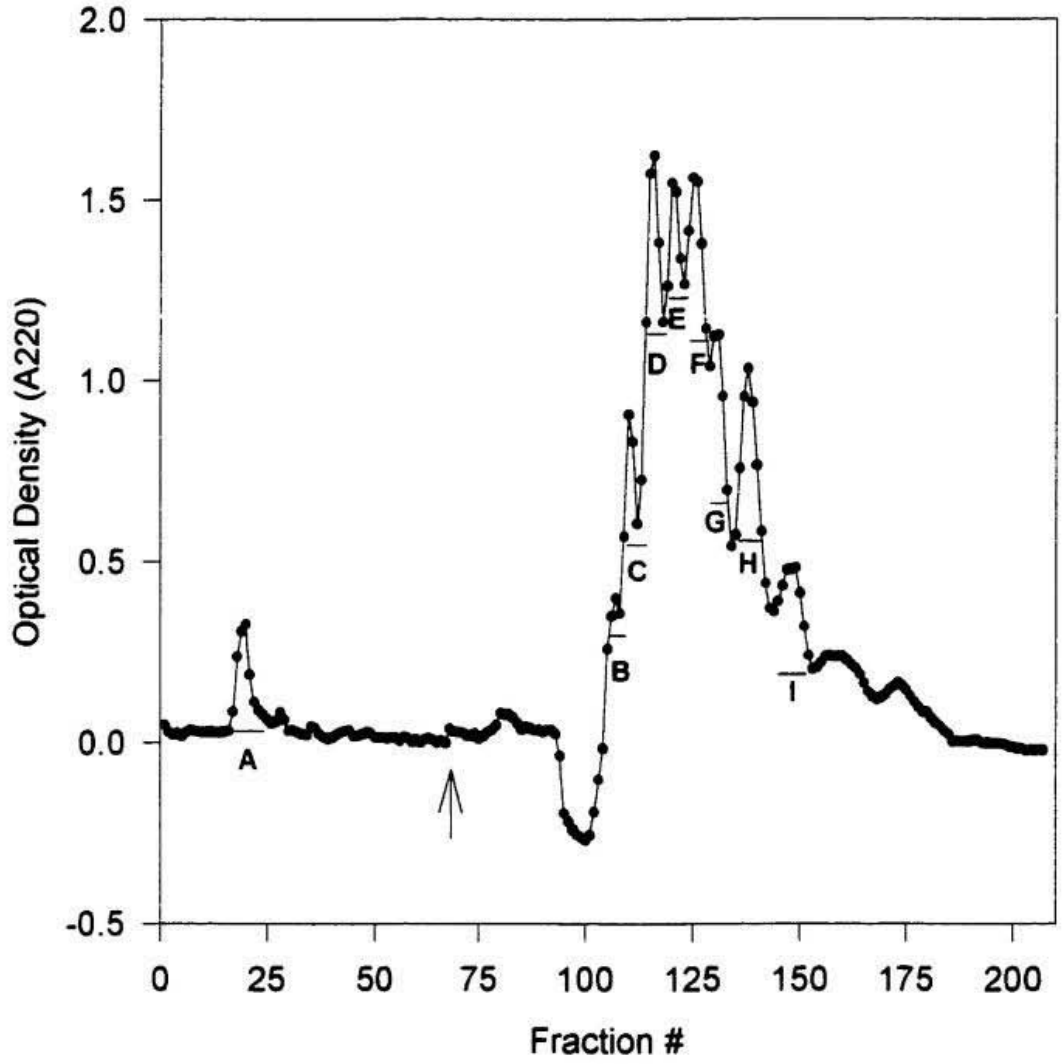


Figure 9: Ion exchange chromatography of combined protamine extracts 1 and 2. A CM-Sephadex C-25 column, 1.5x100 cm, was equilibrated with 0.05M acetate buffer containing 0.5M NaCl, pH 5.8. About 30 mg of protamine was loaded onto the column and washed with 200 mL of this buffer. Fractions were eluted with 0.05M acetate buffer containing 1.5M NaCl, pH 5.8, at a flow rate of 9 mL/h. Fractions of 3 mL were collected and the absorbance measured at 220 nm using a Shimadzu spectrometer. Peaks were pooled as shown above. The arrow indicates the change from equilibration to elution buffer.

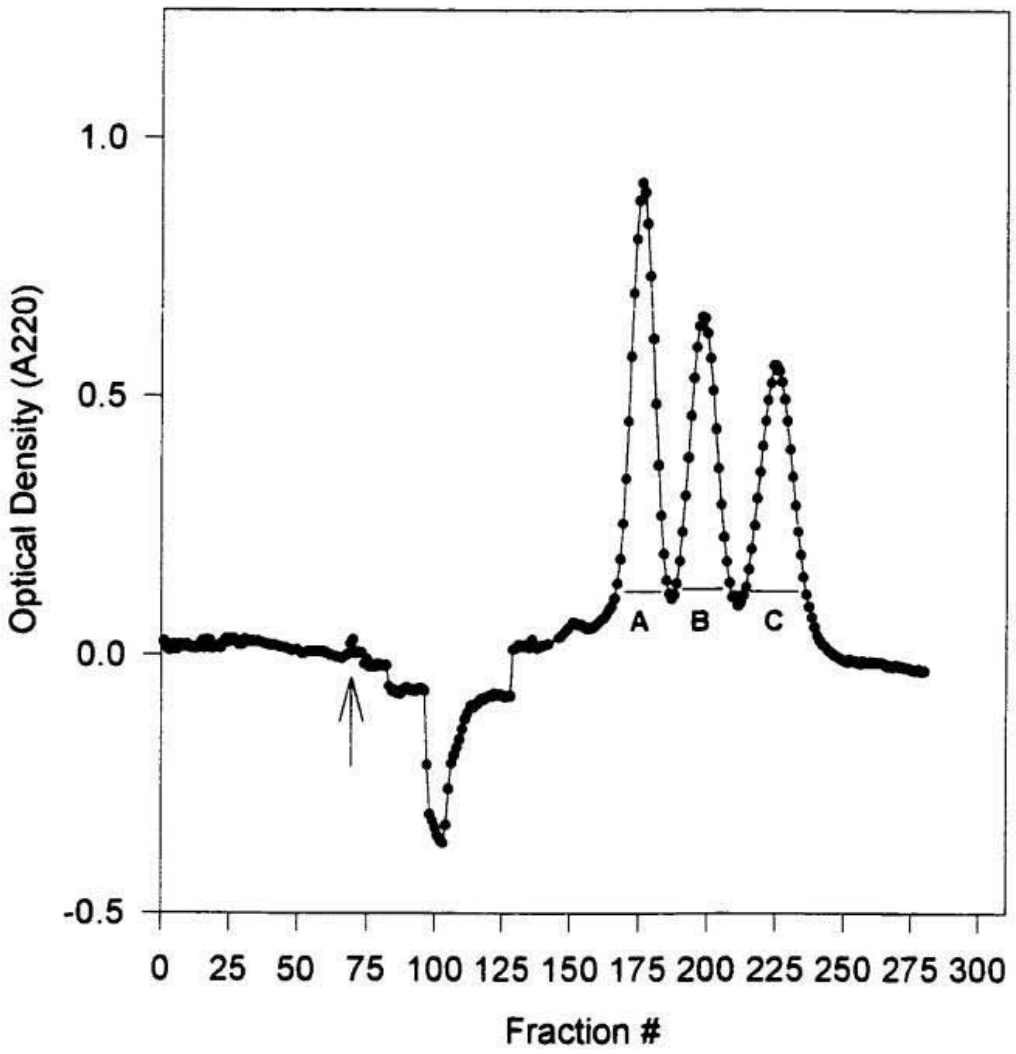


Figure 10: Ion exchange chromatography of Sigma clupeine.
A CM-Sephadex C-25 column, 1.5x100 cm, was equilibrated with 0.05M acetate buffer containing 0.5M NaCl, pH 5.8. About 30 mg of protamine was loaded onto the column and washed with 200 mL of this buffer. Fractions were eluted with 0.05M acetate buffer containing 1.5M NaCl, pH 5.8, at a flow rate of 9 mL/h. Fractions of 3 mL were collected and the absorbance measured at 220 nm using a Shimadzu spectrometer. Peaks were pooled as shown above. The arrow indicates the change from equilibration to elution buffer.

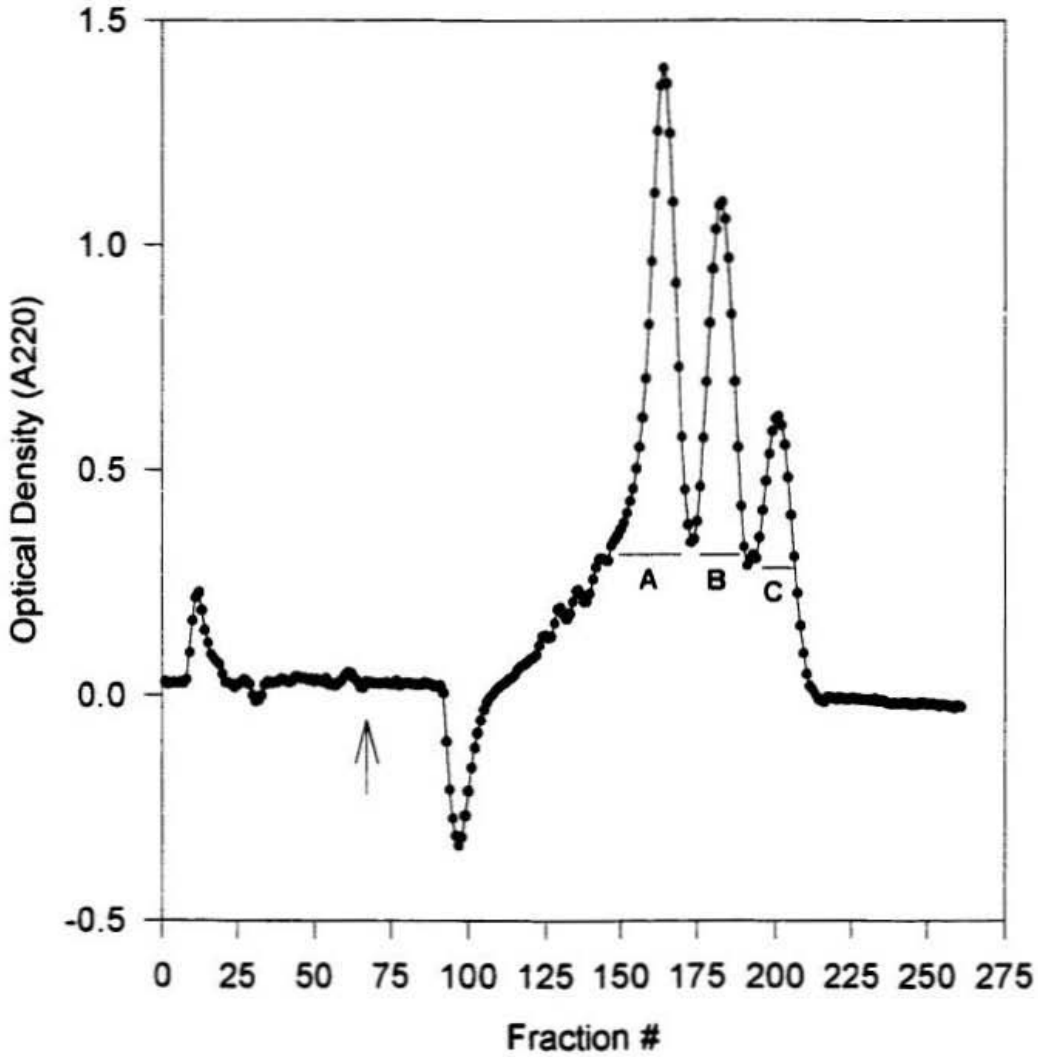


Figure 11: Ion exchange chromatography of isolated protamine oil.
A CM-Sephadex C-25 column, 1.5x100 cm, was equilibrated with 0.05M acetate buffer containing 0.5M NaCl, pH 5.8. About 30 mg of protamine was loaded onto the column and washed with 200 mL of this buffer. Fractions were eluted with 0.05M acetate buffer containing 1.5M NaCl, pH 5.8, at a flow rate of 11 mL/h. Fractions of 3.5 mL were collected and the absorbance measured at 220 nm using a Shimadzu spectrometer. Peaks were pooled as shown above. The arrow indicates the change from equilibration to elution buffer.

3.5 Amino Acid Composition

As can be seen from Table 1, the amino acid compositions of the fractionated components of the isolated protamine oil and clupeine from Sigma correspond very well to the known compositions of the 3 clupeine fractions Y-I, Y-II and Z.

Table 1 : Amino acid compositions determined for the fractionated components of protamine oil (MA, MB, MC) and clupeine from Sigma (SA,SB,SC). The actual compositions of clupeine Y-I, Y-II, and Z are shown for comparison.

	MA	SA	YII	MB	SB	YI	MC	SC	Z
Threonine	0.97 (1)	0.93 (1)	1	1.70 (2)	1.8 (2)	2	---	---	---
Serine	1.97 (2)	1.95 (2)	2	2.68 (3)	2.84 (3)	3	2.83 (3)	2.79 (3)	3
Proline	2.69 (3)	2.23 (2)	3	2.13 (2)	2.09 (2)	2	2.26 (2)	2.28 (2)	2
Alanine	2	2	2	2	2	2	3	3	3
Valine	1.83 (2)	2.59 (3)	2	---	---	---	1.89 (2)	1.79 (2)	2
Arginine	19.74 (20)	19.40 (19)	20	18.99 (19)	20.08 (20)	20	21.02 (21)	21.15 (21)	21
Glycine	---	---	---	0.97 (1)	1.05 (1)	1	---	---	---
Isoleucine	---	---	---	0.86 (1)	0.92 (1)	1	---	---	---
Total	30	29	30	30	31	31	31	31	31

Fractionated protamine samples (1 mL) were hydrolyzed *in vacuo* at 110°C for 24h in 1 mL of 6N HCl containing 0.05% phenol. The HCl was removed under vacuum and the samples reconstituted with pH 2.2 sodium citrate buffer 0.2M Na^+ prior to analysis on a Beckman model 121 MB amino acid analyzer. No corrections were made for losses during hydrolysis. Compositions were calculated relative to the number of alanine residues and rounded to the nearest whole number. These were compared with published amino acid compositions for the 3 clupeine fractions (Ando *et al.*, 1973).

3.6 Mass Spectra

Electrospray mass spectral data for the fractionated components of protamine oil and clupeine (Sigma) are given in Figures 12 - 17. The original spectrum for each component shows many series of peaks of multiply charged ions (see panel A, Figures 12-17). The molecular weight of each series of peaks was determined via maximum entropy processing and these values are shown in panel B of Figures 12-17. The molecular weights of the major and intermediate intensity peaks for each protamine oil fraction, MA, MB and MC, and each fraction of Sigma clupeine, SA, SB and SC, are summarized in Table 2. The range of molecular weights for the minor peaks in each fraction are also given.

Table 2: Molecular weight of the peaks obtained, by maximum entropy processing, for the fractionated components of protamine oil and Sigma clupeine.

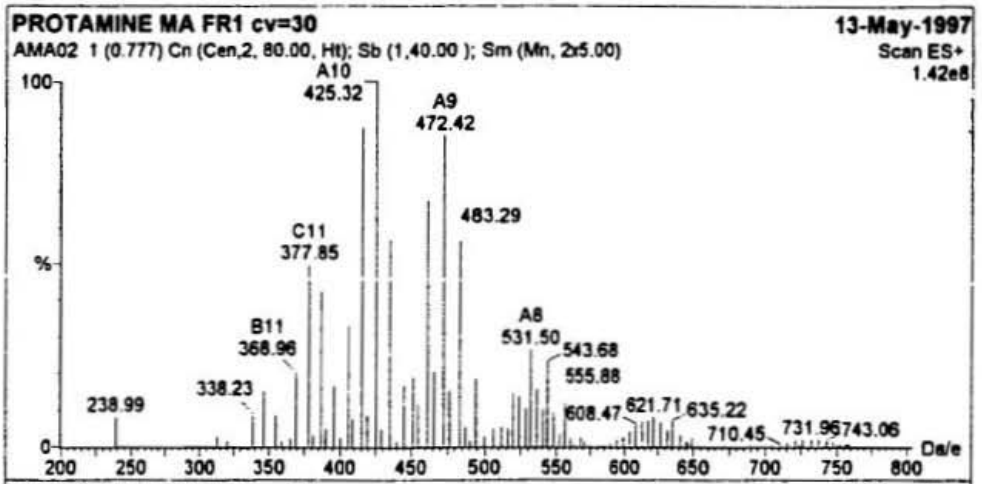
	Molecular Weight (Da.)		
Fraction	Major Peaks	Intermediate Peaks	Minor Peaks *
MA	4150, 4245, 4345		4190 - 5945
MB	4210, 4300, 4310, 4405	4115, 4200,4505	4140 - 5875
MC	4265, 4360, 4450, 4460	4150, 4205, 4310, 4560	4105 - 4960
SA	4150, 4245, 4345	4260, 4360, 4460	4115 - 4925
SB	4150, 4210, 4245, 4310, 4345, 4405	4115, 4440, 4505	4085 - 4895
SC	4210, 4310, 4360, 4460	4150, 4250, 4400, 4550	4010 - 4950

* range of mw's for peaks

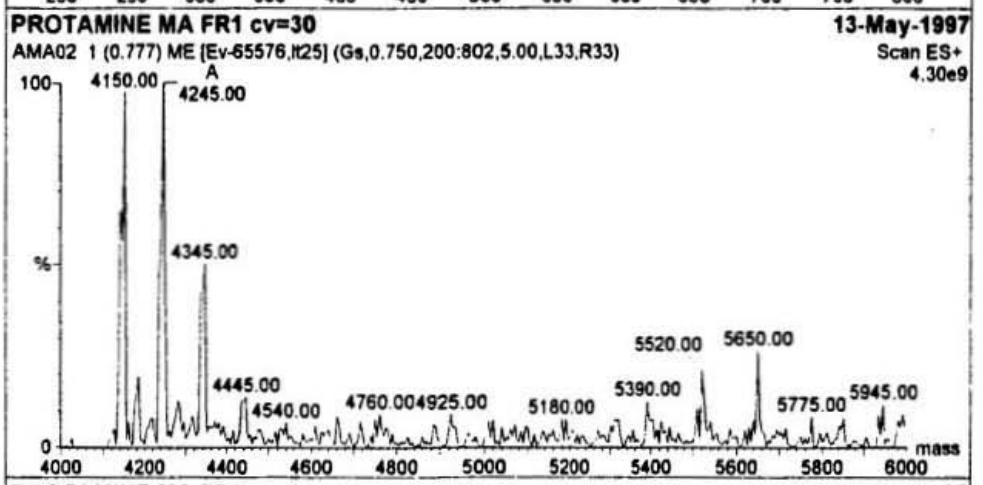
The computer generated mock spectrum of each component is shown in panel C of Figures 12-17. The peaks on the mock spectrum of each component correspond well with the peaks obtained from the original scan (panel A, Figures 12-17).

Figure 12: Electrospray mass spectrum, positive ion mode, of protamine oil fraction A recorded at a cone voltage of 30V.
(A) Different series of peaks obtained from original scan. (B) Molecular weight of each series of peaks determined by Maximum Entropy methods. (C) Computer generated mock spectrum.

(A)


PROTAMINE MA FR1 cv=30
13-May-1997
AMA02 1 (0.777) Cn (Cen,2, 80.00, Ht); Sb (1,40.00); Sm (Mn, 2x5.00)
Scan ES+
1.42e8
A10
425.32
A9
472.42
483.29
C11
377.85
B11
368.96
A8
531.50
543.68
555.88
338.23
238.99
608.47
621.71
635.22
710.45
731.96
743.06
%
Da/e

(B)


PROTAMINE MA FR1 cv=30
13-May-1997
AMA02 1 (0.777) ME [Ev-65576,lt25] (Gs,0.750,200:802,5.00,L33,R33)
Scan ES+
4.30e9
A
4150.00
4245.00
4345.00
4445.00
4540.00
4760.00
4925.00
5180.00
5390.00
5520.00
5650.00
5775.00
5945.00
%
mass

(C)

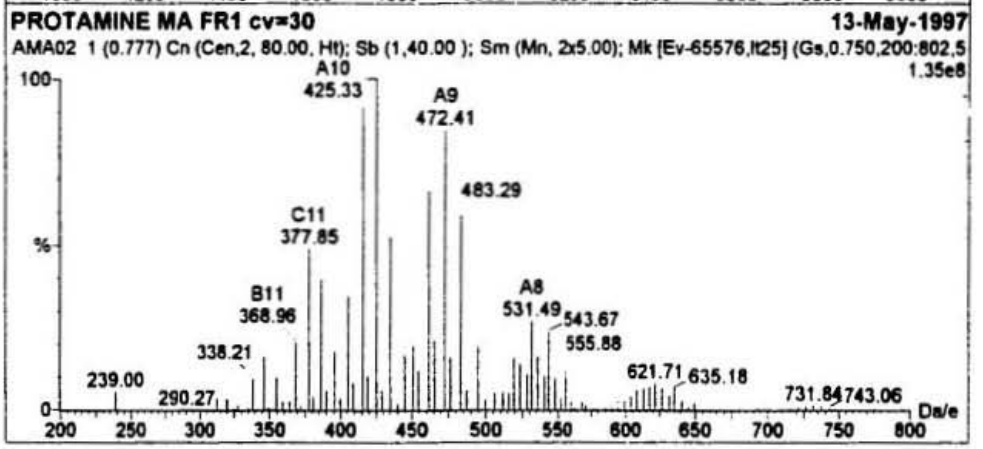
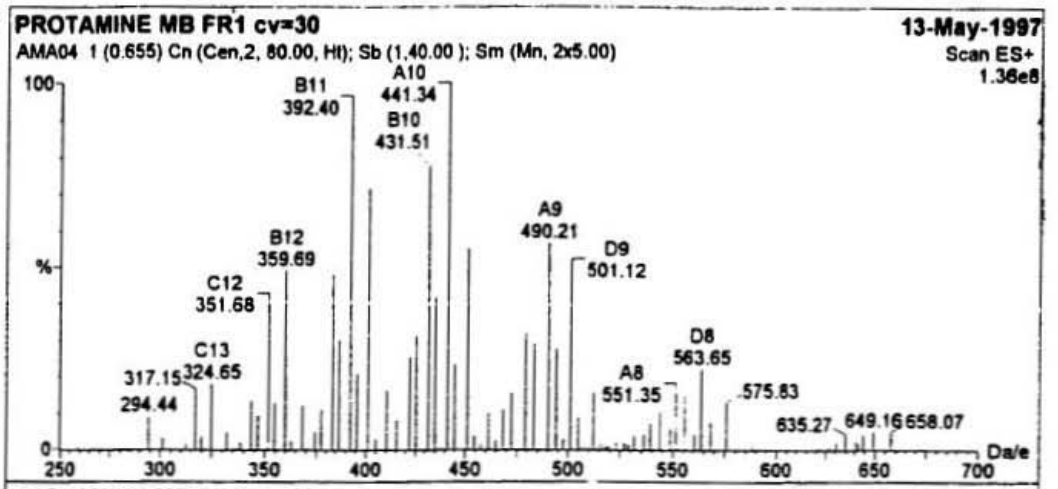
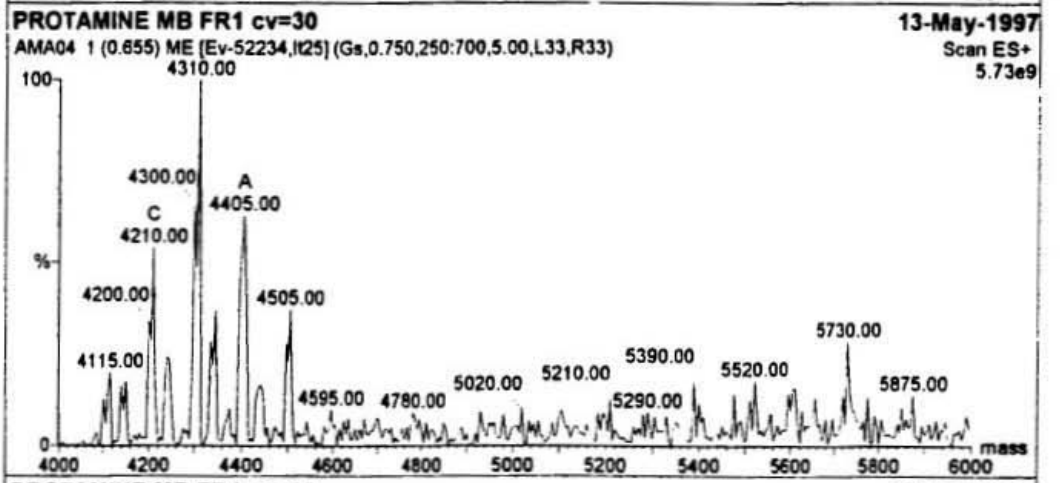

PROTAMINE MA FR1 cv=30
13-May-1997
AMA02 1 (0.777) Cn (Cen,2, 80.00, Ht); Sb (1,40.00); Sm (Mn, 2x5.00); Mk [Ev-65576,lt25] (Gs,0.750,200:802,5
1.35e8
A10
425.33
A9
472.41
483.29
C11
377.85
B11
368.96
A8
531.49
543.67
555.88
338.21
239.00
290.27
621.71
635.18
731.84
743.06
%
Da/e

Figure 13: Electrospray mass spectrum, positive ion mode, of protamine oil fraction B recorded at a cone voltage of 30V.
(A) Different series of peaks obtained from original scan. (B) Molecular weight of each series of peaks determined by Maximum Entropy methods. (C) Computer generated mock spectrum.

(A)


PROTAMINE MB FR1 cv=30
13-May-1997
AMA04 1 (0.655) Cn (Cen,2, 80.00, Ht); Sb (1,40.00); Sm (Mn, 2x5.00)
Scan ES+
1.36e8
B11
392.40
A10
441.34
B10
431.51
A9
490.21
B12
359.69
D9
501.12
C12
351.68
D8
563.65
C13
324.65
317.15
294.44
A8
551.35
575.83
635.27
649.16
658.07
Da/e

(B)


PROTAMINE MB FR1 cv=30
13-May-1997
AMA04 1 (0.655) ME [Ev-52234,lt25] (Gs,0.750,250:700,5.00,L33,R33)
Scan ES+
5.73e9
4310.00
4300.00
A
4405.00
C
4210.00
4200.00
4505.00
4115.00
5730.00
5390.00
5520.00
5020.00
5210.00
5875.00
4595.00
4780.00
5290.00
mass

(C)

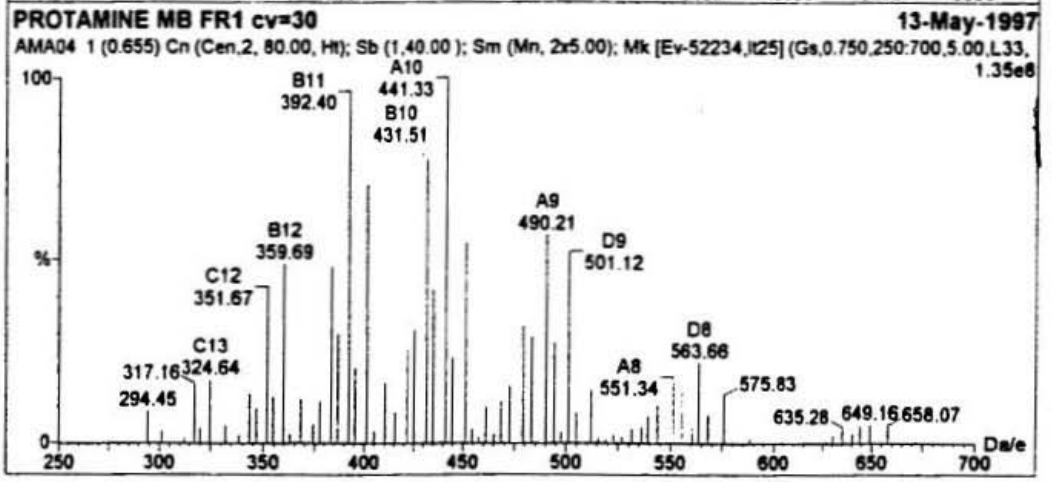

PROTAMINE MB FR1 cv=30
13-May-1997
AMA04 1 (0.655) Cn (Cen,2, 80.00, Ht); Sb (1,40.00); Sm (Mn, 2x5.00); Mk [Ev-52234,lt25] (Gs,0.750,250:700,5.00,L33,
1.35e8
B11
392.40
A10
441.33
B10
431.51
A9
490.21
B12
359.69
D9
501.12
C12
351.67
D8
563.66
C13
324.64
317.16
294.45
A8
551.34
575.83
635.28
649.16
658.07
Da/e

Figure 14: Electrospray mass spectrum, positive ion mode, of protamine oil fraction C recorded at a cone voltage of 30V.
(A) Different series of peaks obtained from original scan. (B) Molecular weight of each series of peaks determined by Maximum Entropy methods.
(C) Computer generated mock spectrum.

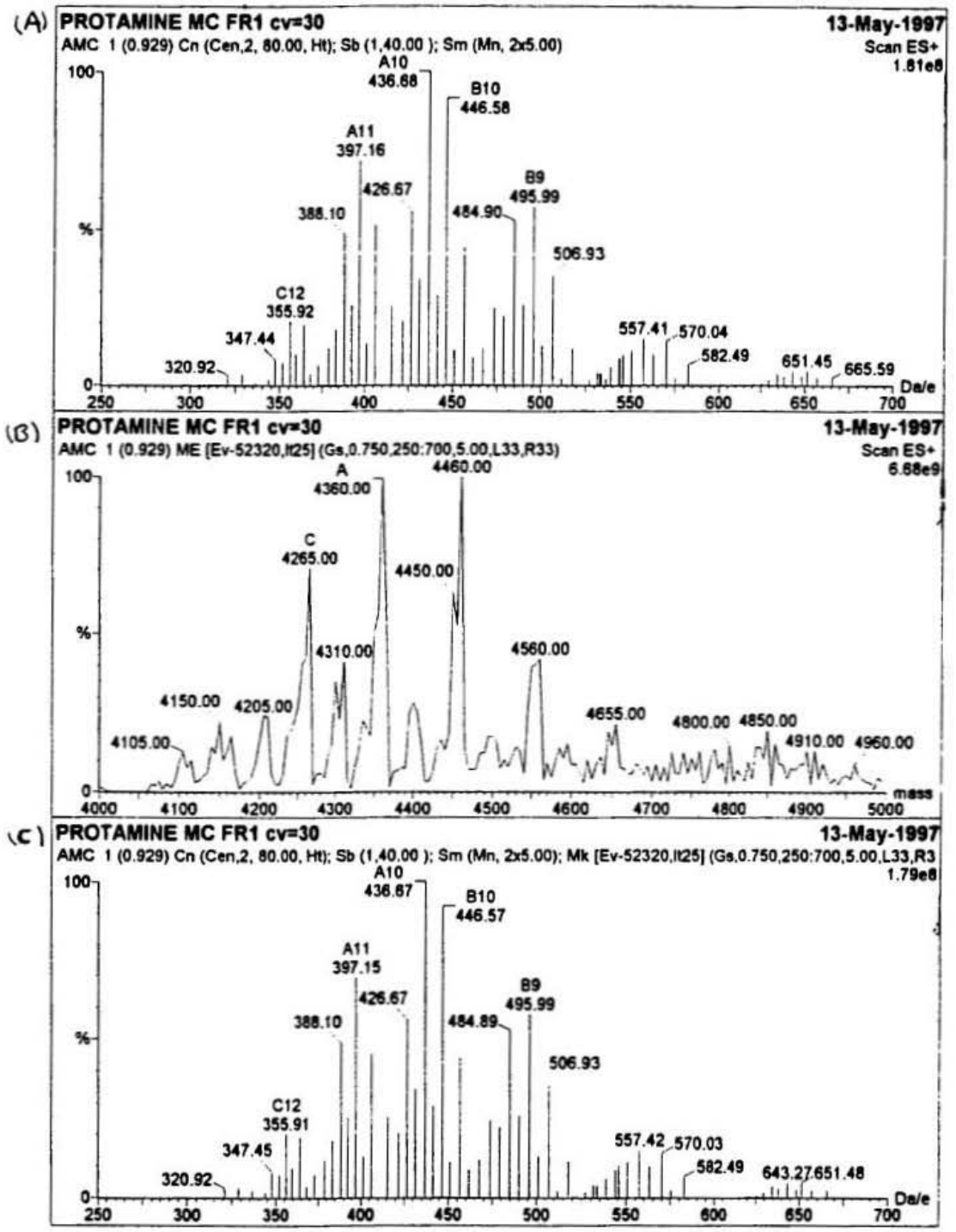

(A)
PROTAMINE MC FR1 cv=30
13-May-1997
AMC 1 (0.929) Cn (Cen,2, 80.00, Ht); Sb (1,40.00); Sm (Mn, 2x5.00)
Scan ES+
1.81e8
A10
436.68
B10
446.58
A11
397.16
426.67
B9
495.99
388.10
484.90
506.93
C12
355.92
347.44
320.92
557.41
570.04
582.49
651.45
665.59
Da/e
(B)
PROTAMINE MC FR1 cv=30
13-May-1997
AMC 1 (0.929) ME [Ev-52320,lt25] (Gs,0.750,250:700,5.00,L33,R33)
Scan ES+
6.68e9
A
4360.00
4460.00
C
4265.00
4450.00
4310.00
4560.00
4150.00
4205.00
4655.00
4800.00
4850.00
4105.00
4910.00
4960.00
mass
(C)
PROTAMINE MC FR1 cv=30
13-May-1997
AMC 1 (0.929) Cn (Cen,2, 80.00, Ht); Sb (1,40.00); Sm (Mn, 2x5.00); Mk [Ev-52320,lt25] (Gs,0.750,250:700,5.00,L33,R3
1.79e8
A10
436.67
B10
446.57
A11
397.15
426.67
B9
495.99
388.10
484.89
506.93
C12
355.91
347.45
320.92
557.42
570.03
582.49
643.27
651.48
Da/e

Figure 15: Electrospray mass spectrum, positive ion mode, of Sigma clupeine fraction A recorded at a cone voltage of 30V.
(A) Different series of peaks obtained from original scan. (B) Molecular weight of each series of peaks determined by Maximum Entropy methods. (C) Computer generated mock spectrum.

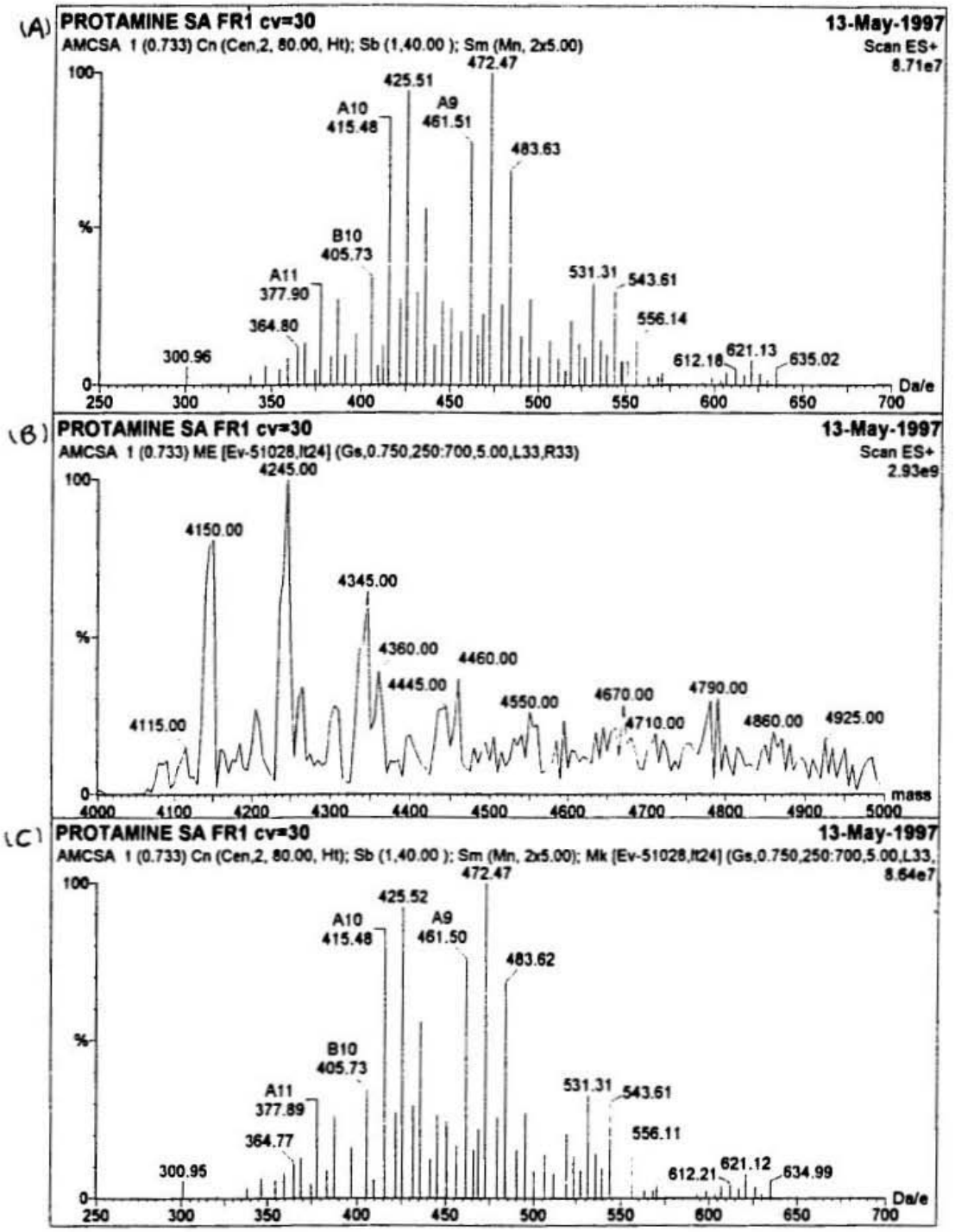

(A)
PROTAMINE SA FR1 cv=30
13-May-1997
AMCSA 1 (0.733) Cn (Cen,2, 80.00, Ht); Sb (1,40.00); Sm (Mn, 2x5.00)
Scan ES+
8.71e7
100
%
0
425.51
472.47
A10
415.48
A9
461.51
483.63
B10
405.73
A11
377.90
531.31
543.61
364.80
556.14
300.96
612.18
621.13
635.02
Da/e
250
300
350
400
450
500
550
600
650
700
(B)
PROTAMINE SA FR1 cv=30
13-May-1997
AMCSA 1 (0.733) ME [Ev-51028,lt24] (Gs,0.750,250:700,5.00,L33,R33)
Scan ES+
2.93e9
100
%
0
4245.00
4150.00
4345.00
4360.00
4460.00
4445.00
4550.00
4670.00
4790.00
4115.00
4710.00
4860.00
4925.00
mass
4000
4100
4200
4300
4400
4500
4600
4700
4800
4900
5000
(C)
PROTAMINE SA FR1 cv=30
13-May-1997
AMCSA 1 (0.733) Cn (Cen,2, 80.00, Ht); Sb (1,40.00); Sm (Mn, 2x5.00); Mk [Ev-51028,lt24] (Gs,0.750,250:700,5.00,L33,
8.64e7
100
%
0
472.47
425.52
A10
415.48
A9
461.50
483.62
B10
405.73
A11
377.89
531.31
543.61
364.77
556.11
300.95
612.21
621.12
634.99
Da/e
250
300
350
400
450
500
550
600
650
700

Figure 16: Electrospray mass spectrum, positive ion mode, of Sigma clupeine fraction B recorded at a cone voltage of 30V.
(A) Different series of peaks obtained from original scan. (B) Molecular weight of each series of peaks determined by Maximum Entropy methods. (C) Computer generated mock spectrum.

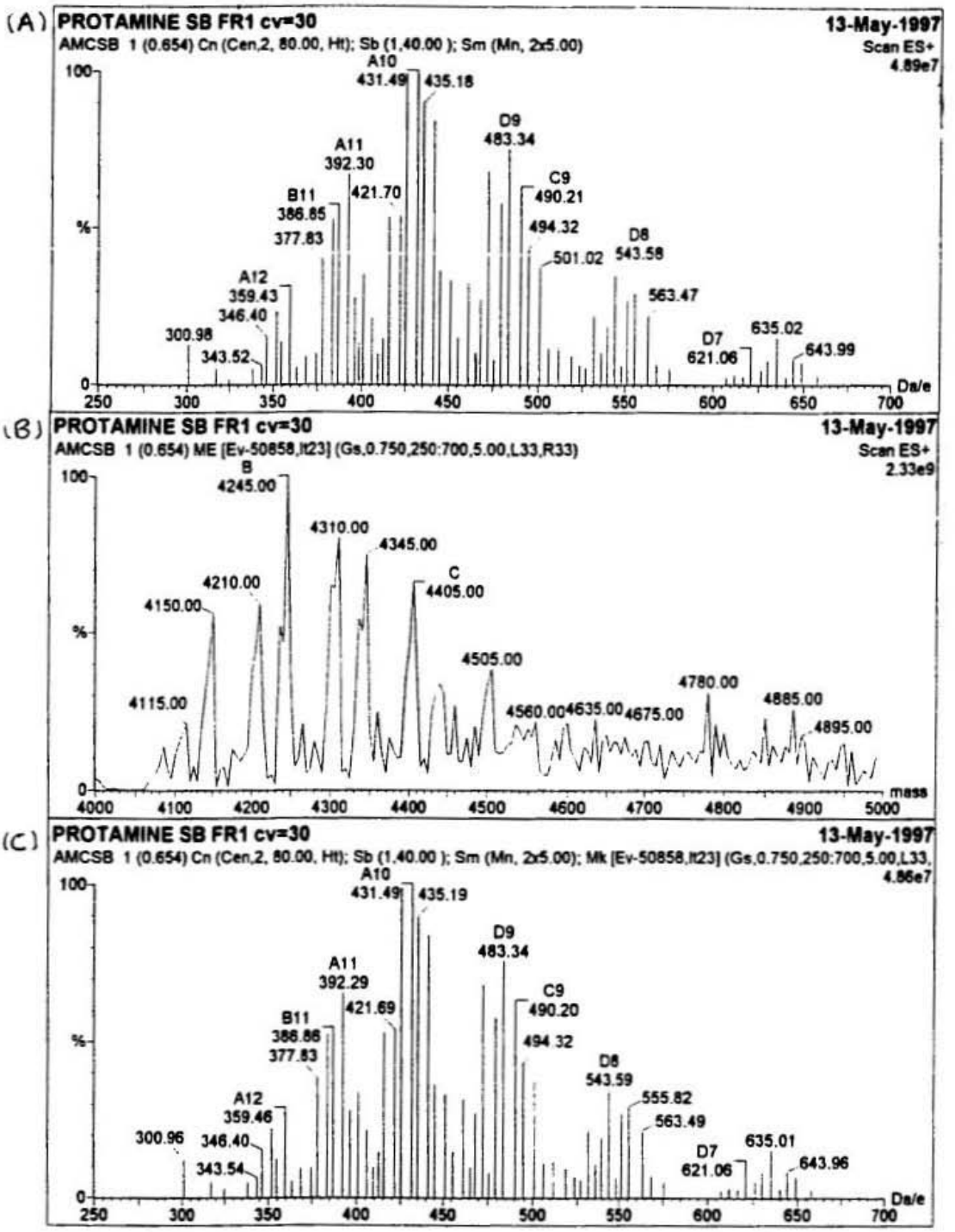
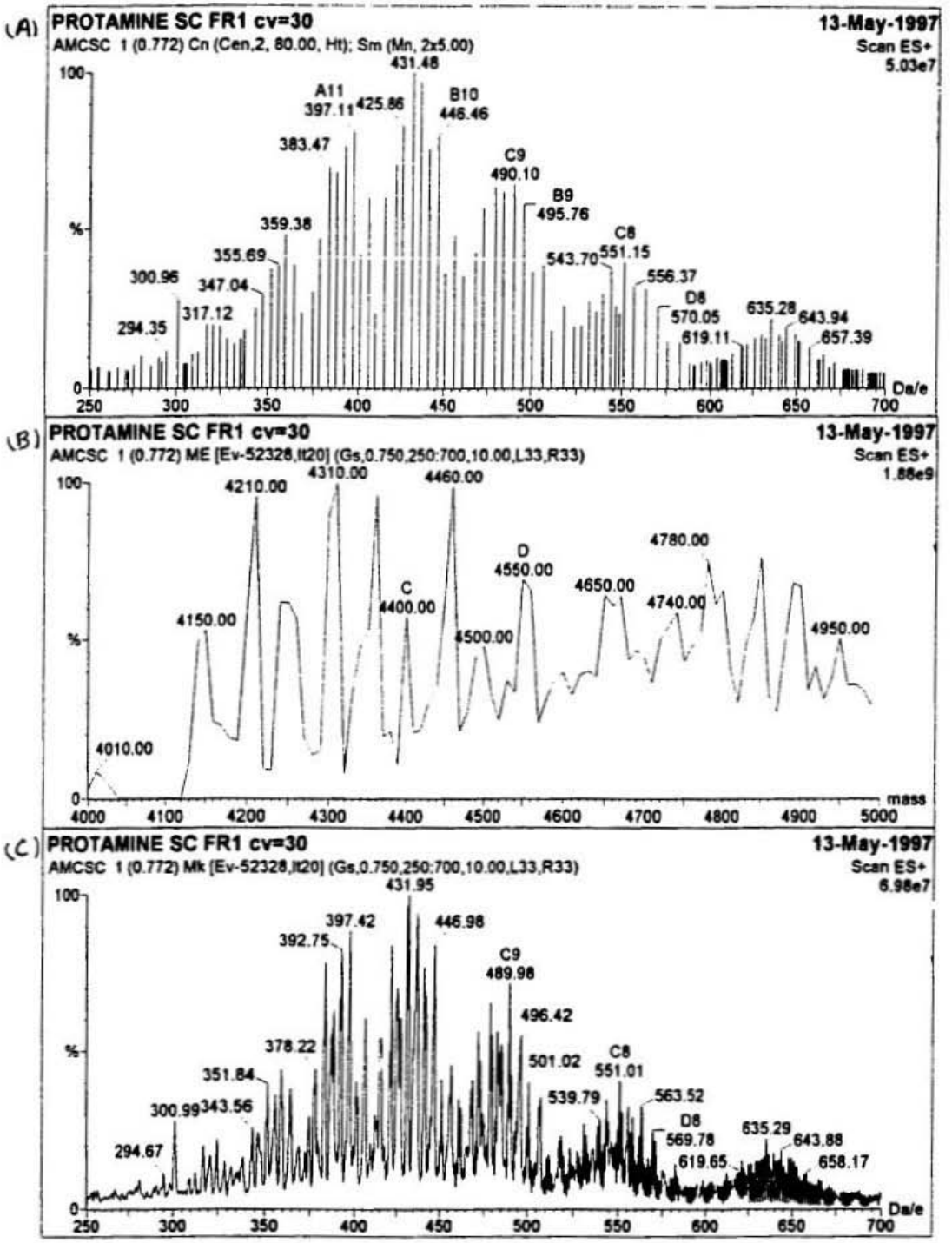

(A)
PROTAMINE SB FR1 cv=30
13-May-1997
AMCSB 1 (0.654) Cn (Cen,2, 80.00, Ht); Sb (1,40.00); Sm (Mn, 2x5.00)
Scan ES+
4.89e7
A10
431.49
435.18
D9
483.34
A11
392.30
421.70
C9
490.21
B11
386.85
377.83
494.32
D8
543.58
501.02
A12
359.43
346.40
563.47
300.98
343.52
635.02
D7
621.06
643.99
Da/e
(B)
PROTAMINE SB FR1 cv=30
13-May-1997
AMCSB 1 (0.654) ME [Ev-50858,It23] (Gs,0.750,250:700,5.00,L33,R33)
Scan ES+
2.33e9
B
4245.00
4310.00
4345.00
4210.00
C
4405.00
4150.00
4505.00
4780.00
4115.00
4560.00
4635.00
4675.00
4885.00
4895.00
mass
(C)
PROTAMINE SB FR1 cv=30
13-May-1997
AMCSB 1 (0.654) Cn (Cen,2, 80.00, Ht); Sb (1,40.00); Sm (Mn, 2x5.00); Mk [Ev-50858,It23] (Gs,0.750,250:700,5.00,L33,
4.86e7
A10
431.49
435.19
D9
483.34
A11
392.29
421.69
C9
490.20
B11
386.86
377.83
494.32
D8
543.59
555.82
A12
359.46
563.49
300.96
346.40
635.01
D7
621.06
643.96
343.54
Da/e

Figure 17: Electrospray mass spectrum, positive ion mode, of Sigma clupeine fraction C recorded at a cone voltage of 30V.
(A) Different series of peaks obtained from original scan. (B) Molecular weight of each series of peaks determined by Maximum Entropy methods. (C) Computer generated mock spectrum.


(A)
PROTAMINE SC FR1 cv=30
AMCSC 1 (0.772) Cn (Cen,2, 80.00, Ht); Sm (Mn, 2x5.00)
13-May-1997
Scan ES+
5.03e7
100
%
0
431.48
A11
397.11
425.86
B10
446.46
383.47
C9
490.10
B9
495.76
359.38
C8
551.15
355.69
543.70
556.37
300.96
347.04
D8
570.05
635.28
643.94
317.12
294.35
619.11
657.39
250
300
350
400
450
500
550
600
650
700
Da/e
(B)
PROTAMINE SC FR1 cv=30
AMCSC 1 (0.772) ME [Ev-52328,It20] (Gs,0.750,250:700,10.00,L33,R33)
13-May-1997
Scan ES+
1.88e9
100
%
0
4210.00
4310.00
4460.00
4780.00
D
4550.00
4650.00
C
4400.00
4740.00
4150.00
4500.00
4950.00
4010.00
4000
4100
4200
4300
4400
4500
4600
4700
4800
4900
5000
mass
(C)
PROTAMINE SC FR1 cv=30
AMCSC 1 (0.772) Mk [Ev-52328,It20] (Gs,0.750,250:700,10.00,L33,R33)
13-May-1997
Scan ES+
6.98e7
100
%
0
431.95
397.42
446.98
392.75
C9
489.98
496.42
378.22
501.02
C8
551.01
351.84
539.79
563.52
300.99
343.56
D8
635.29
569.78
643.88
294.67
619.65
658.17
250
300
350
400
450
500
550
600
650
700
Da/e

3.7 Antimicrobial Activity

The lowest concentration of protamine resulting in an optical density, at 625 nm, of < 0.100 after the appropriate incubation period, or < $5x10^5$ CFU/mL for *L. monocytogenes*, was designated as the MIC or minimum inhibitory concentration. The MIC's for isolated protamine oil and clupeine from Sigma are given in Table 3.

Table 3: Summary of MIC values of protamine oil and clupeine (Sigma) against 9 bacterial species.

	Minimum Inhibitory Concentration μg/mL			
	Isolated Protamine		Sigma Clupeine	
	Trial 1	Trial 2	Trial 1	Trial 2
Bacillus subtilis	50	50	?[A]	50
Listeria monocytogenes	800	800	800	800
Staphylococcus aureus	25	25	?[A]	25
Aeromonas salmonicida	1000	800	1000	1000
Aeromonas sobria	>400[B]	400	>400	400
Escherichia coli	50	25	?[A]	50
Pseudomonas aeruginosa	100	200	100	200
Pseudomonas fluorescens	200	200	>400	200
Salmonella typhimurium	100	50	?[A]	100
Vibrio anguillarum	25	50	50	25
Yersinia ruckerii	>1000	1000	1000	1000

[A] - OD of 0.1-0.2, and did not decrease with increased amount of protamine.
[B] -1 of duplicate 400

Figures 18 to 28 are the graphs of growth inhibition determined from the optical density values. The actual OD readings are given in the appendix (see Tables A.9 to A.19). In most cases, there was a decrease in optical density, as compared to control tubes lacking protamine, of 90% or greater at protamine concentrations at, or above, the MIC. For *Vibrio anguillarum* (see Figure 27) there was a decrease in OD of about 80% for trial 1 but this is the result of lower control OD values in this trial (see Table A.18, appendix). For trial 1 with Sigma clupeine, *Bacillus subtilis* (Figure 18), *Staphylococcus aureus* (Figure 20), and *Escherichia coli* (Figure 23) each had about a 90% decrease in OD at the MIC found for trial 2, however this dropped to 84% or less in tubes with a higher protamine concentration. With *Salmonella typhimurium* in trial 1, there was a decrease in OD of about 80%, at the MIC found for trial 2, and this remained about constant with increased in protamine concentration (see Figure 26). These 4 instances are marked by a $?^{A}$ in Table 2. For *Aeromonas sobria*, trial 1, the greatest percentage decrease in OD was about 77% and 53% for isolated protamine and Sigma clupeine respectively (see Figure 22). For *Pseudomonas fluorescens*, trial 1, the percentage decrease in OD was about 91% which is very slightly below the level of the others at their MIC's (see Figure 25). With *Yersinia ruckerii*, trial 1, the greatest percentage decrease in OD for isolated protamine was about 75%.

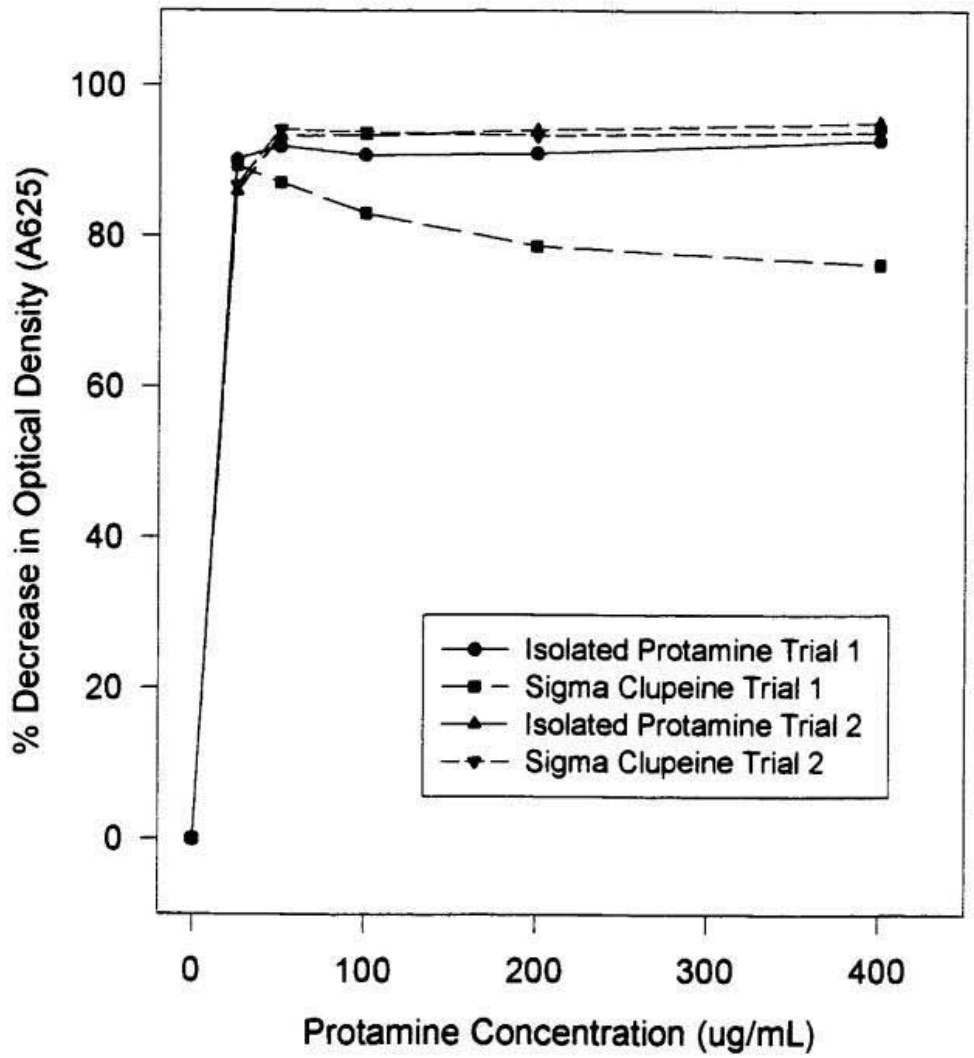


Figure 18: Effect of different concentrations of isolated protamine and Sigma clupeine on the growth of *Bacillus subtilis*.
A standardized inoculum of *B. subtilis* was prepared and 0.1 mL aliquots used to inoculate 2.4 mL of Mueller-Hinton Broth containing different concentrations of isolated protamine or clupeine (Sigma) in 16x125 mm test tubes. The initial level of bacteria was 9.63×10^4 CFU/mL for both trial 1 and trial 2. Tubes were incubated at 30°C for 22 h in trial 1 and 24 h in trial 2 and optical densities measured in a Shimadzu spectrophotometer.

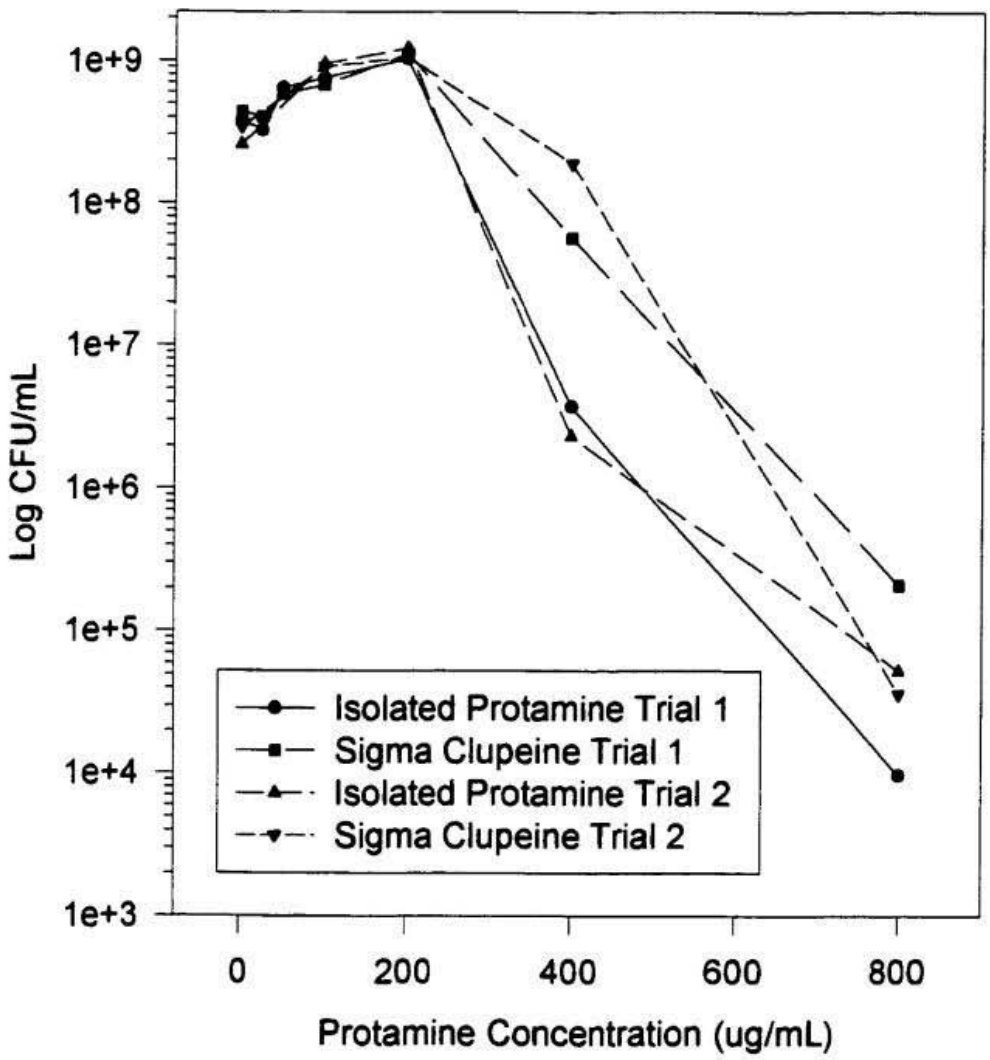


Figure 19: Number of surviving *Listeria monocytogenes* 4b after treatment with different concentrations of isolated protamine or Sigma clupeine. A standardized inoculum of *L. monocytogenes* was prepared and 0.1 mL aliquots used to inoculate 2.4 mL of *Listeria* enrichment broth containing different concentrations of isolated protamine or clupeine (Sigma) in 16x125 mm test tubes. The initial level of bacteria was 3.55×10^5 CFU/mL for trial 1 and 5.31×10^5 CFU/mL for trial 2. Tubes were incubated at 37°C for 24 h and optical densities measured in a Shimadzu spectrophotometer.

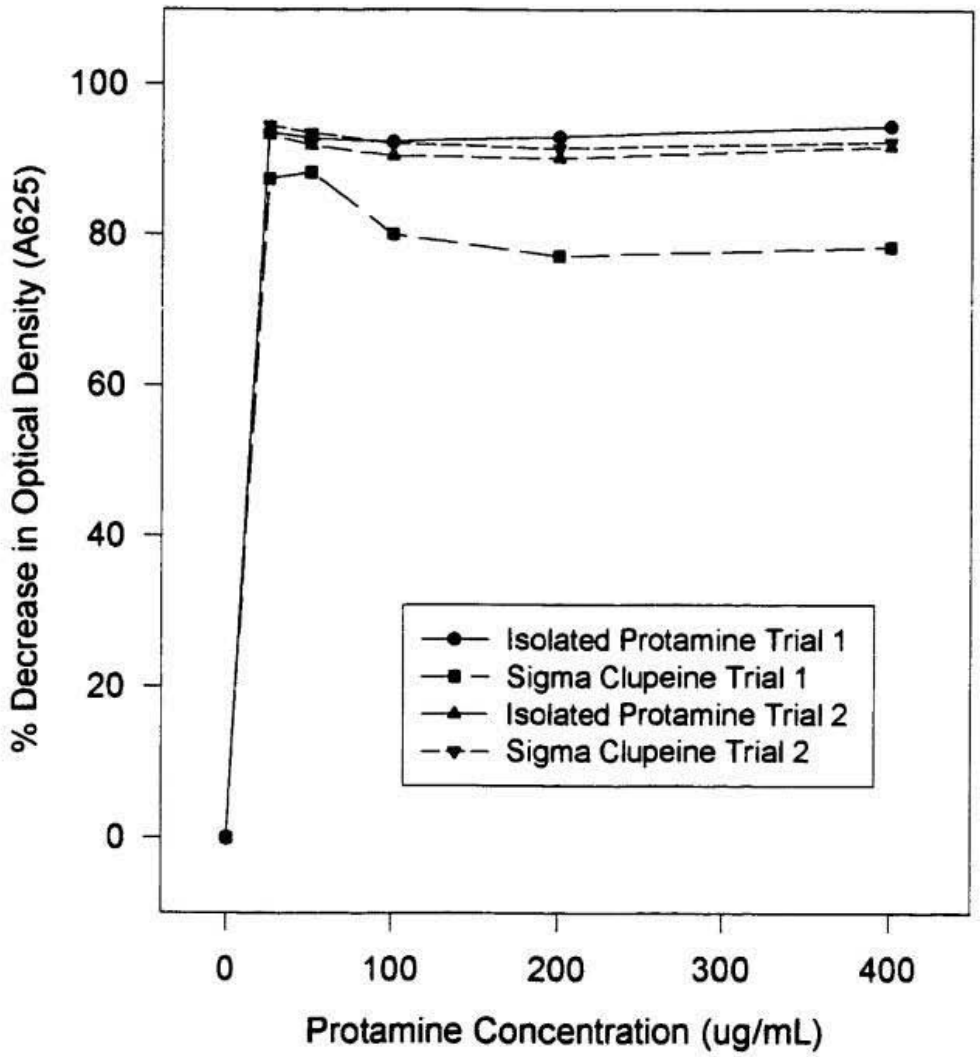


Figure 20: Effect of different concentrations of isolated protamine and Sigma clupeine on the growth of *Staphylococcus aureus*.
A standardized inoculum of *S. aureus* was prepared and 0.1 mL aliquots used to inoculate 2.4 mL of MHB containing different concentrations of isolated protamine or clupeine (Sigma) in 16x125 mm test tubes. The initial level of bacteria was 6.90×10^5 CFU/mL for trial 1 and 6.71×10^5 CFU/mL for trial 2. Tubes were incubated at 37°C for 22 h and optical densities measured in a Spectronic 601 (trial 1). Due to technical problems in trial 2, tubes were refrigerated for 24 h, then optical densities measured in the Shimadzu spectrophotometer at 625 nm.

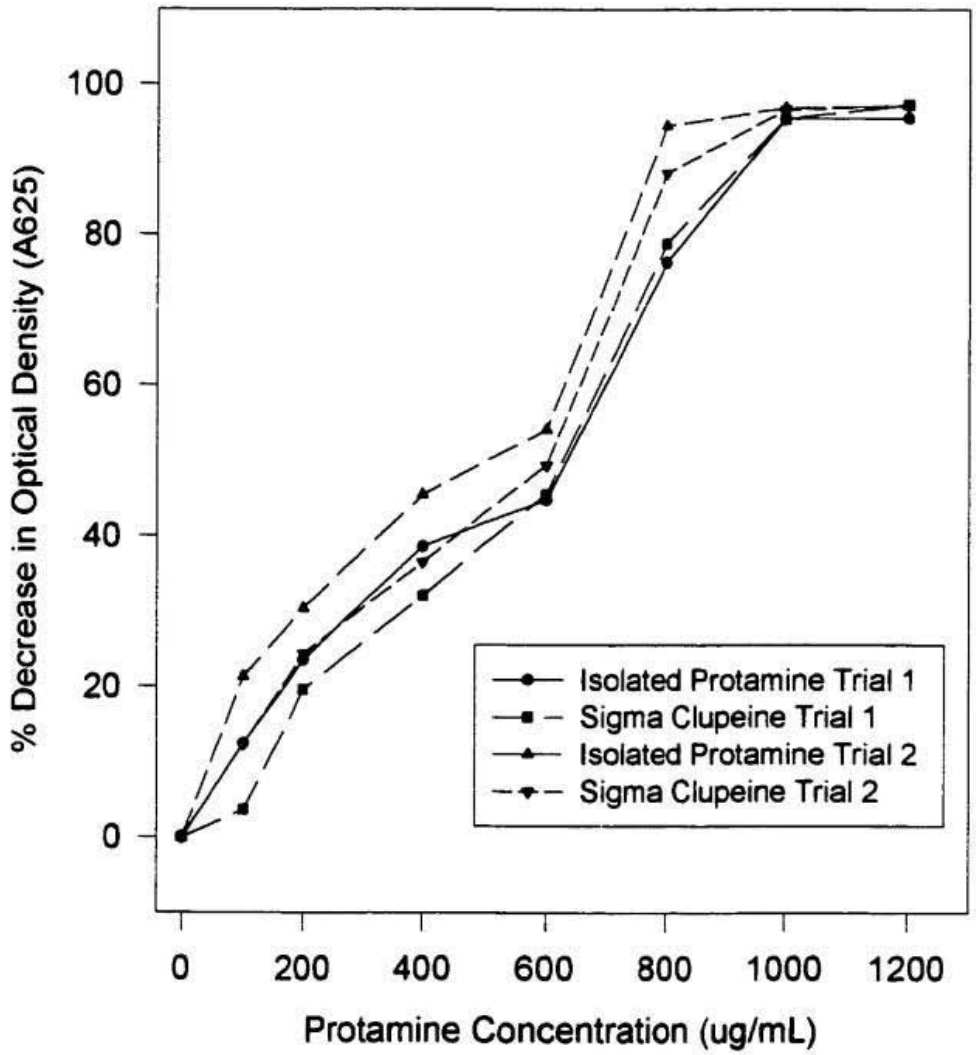


Figure 21: Effect of different concentrations of isolated protamine and Sigma clupeine on the growth of *Aeromonas salmonicida*.
A standardized inoculum of *A. salmonicida* was prepared and 0.1 mL aliquots used to inoculate 2.4 mL of MHB containing different concentrations of isolated protamine or clupeine (Sigma) in 16x125 mm test tubes. The initial level of bacteria was 3.70×10^5 CFU/mL for trial 1 and 4.64×10^5 CFU/mL for trial 2. Tubes were incubated at room temperature for 48 h and optical densities measured in a Shimadzu spectrophotometer at 625 nm.

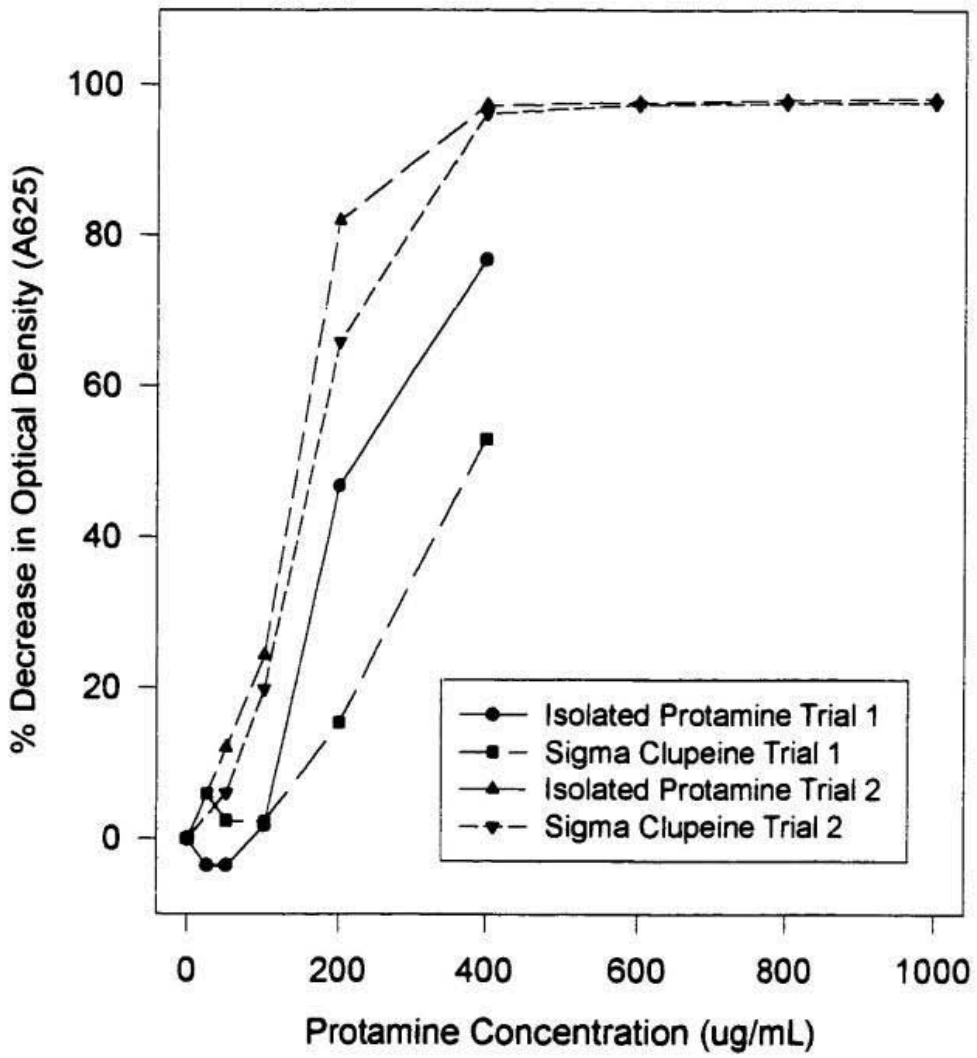


Figure 22: Effect of different concentrations of isolated protamine and Sigma clupeine on the growth of *Aeromonas sobria*.
A standardized inoculum of *A. sobria* was prepared and 0.1 mL aliquots used to inoculate 2.4 mL of MHB containing different concentrations of isolated protamine or clupeine (Sigma) in 16x125 mm test tubes. The initial level of bacteria was 1.19×10^5 CFU/mL for trial 1 and 1.46×10^5 CFU/mL for trial 2. Tubes were incubated at room temperature for 24 h and optical densities measured in a Spectronic 601 (trial 1) and a Shimadzu (trial 2) spectrophotometer at 625 nm.

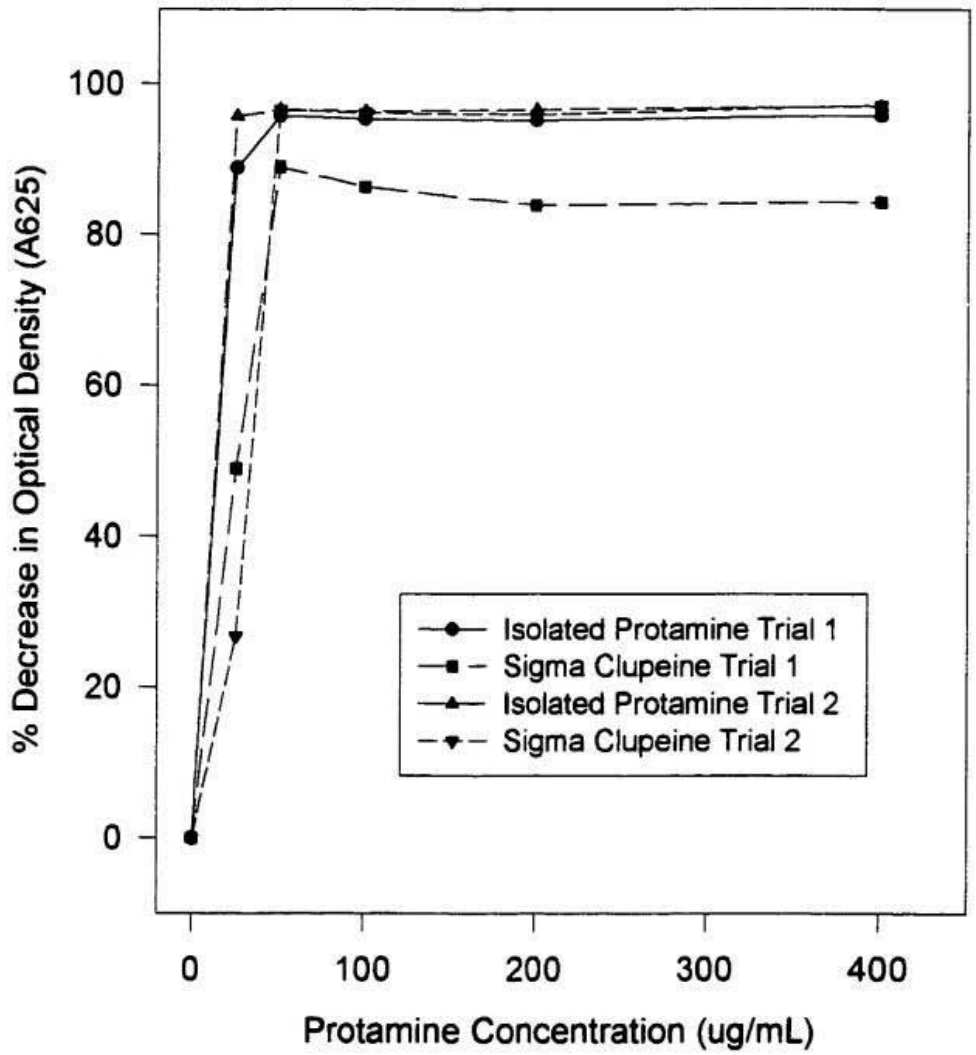


Figure 23: Effect of different concentrations of isolated protamine and Sigma clupeine on the growth of *Escherichia coli*.
A standardized inoculum of *E. coli* was prepared and 0.1 mL aliquots used to inoculate 2.4 mL of MHB containing different concentrations of isolated protamine or clupeine (Sigma) in 16x125 mm test tubes. The initial level of bacteria was 3.95×10^5 CFU/mL for trial 1 and 3.30×10^5 CFU/mL for trial 2. Tubes were incubated at 37°C for 20 h in trial 1 and 24 h in trial 2 and optical densities measured in a Shimadzu spectrophotometer at 625 nm.

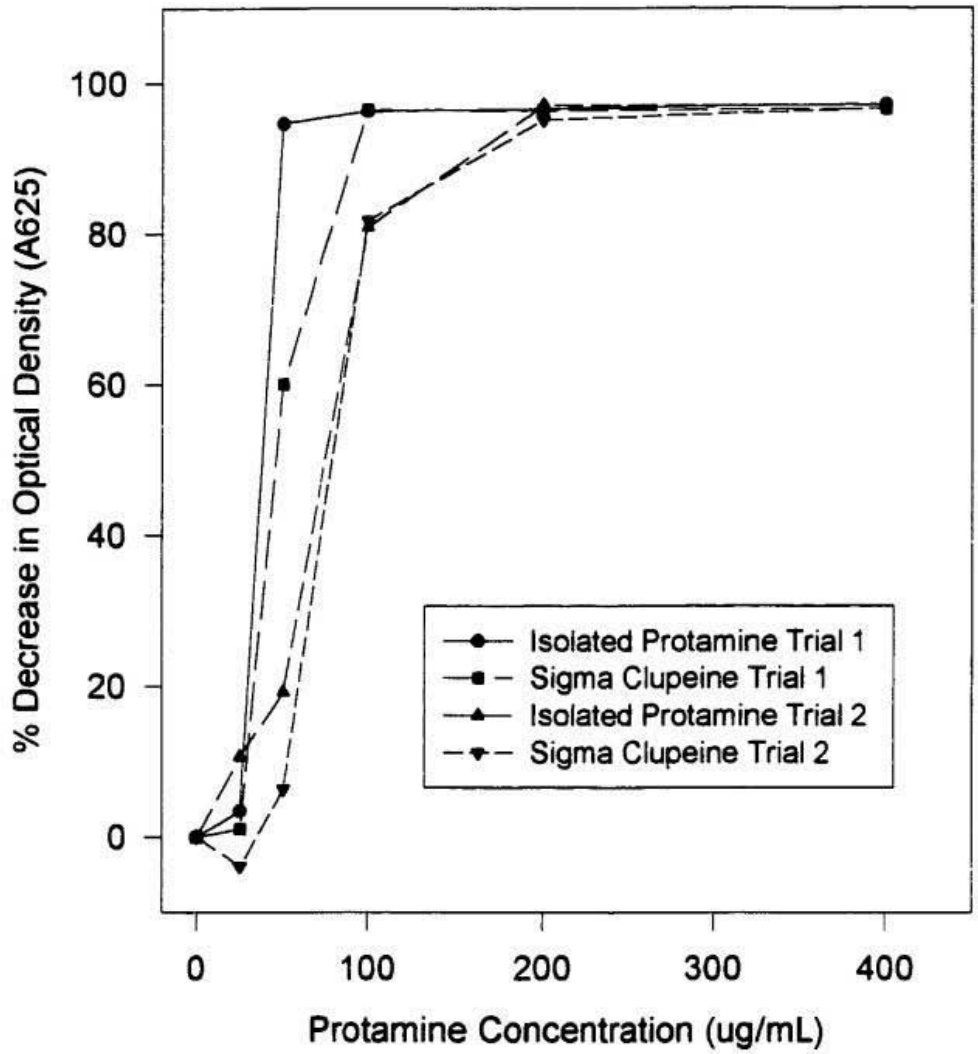


Figure 24: Effect of different concentrations of isolated protamine and Sigma clupeine on the growth of *Pseudomonas aeruginosa*.
A standardized inoculum of *P. aeruginosa* was prepared and 0.1 mL aliquots used to inoculate 2.4 mL of MHB containing different concentrations of isolated protamine or clupeine (Sigma) in 16x125 mm test tubes. The initial level of bacteria was 2.51×10^5 CFU/mL for trial 1 and 2.60×10^5 CFU/mL for trial 2. Tubes were incubated at 37°C for 24 h and optical densities measured in a Shimadzu spectrophotometer at 625 nm.

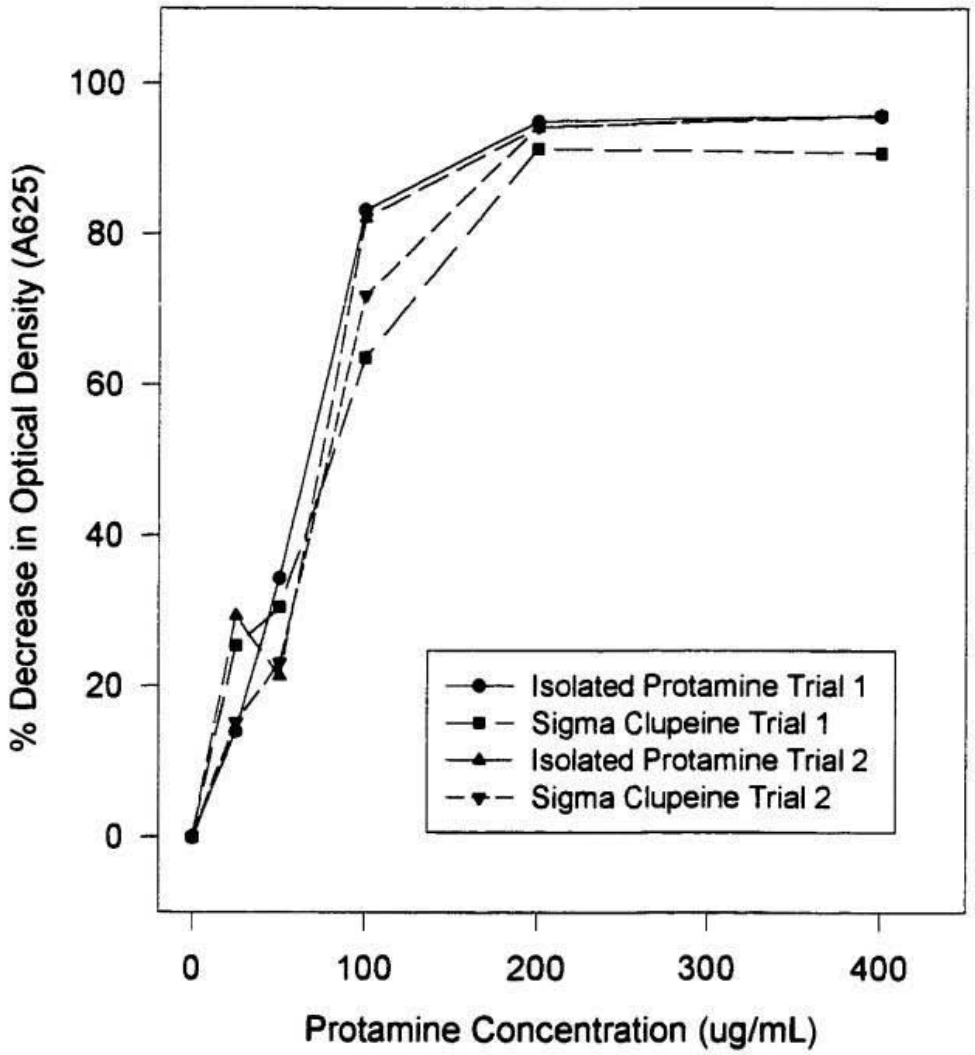


Figure 25: Effect of different concentrations of isolated protamine and Sigma clupeine on the growth of *Pseudomonas fluorescens*.
A standardized inoculum of *P. fluorescens* was prepared and 0.1 mL aliquots used to inoculate 2.4 mL of MHB containing different concentrations of isolated protamine or clupeine (Sigma) in 16x125 mm test tubes. The initial level of bacteria was 1.43×10^5 CFU/mL for trial 1 and 2.28×10^5 CFU/mL for trial 2. Tubes were incubated at room temperature for 24 h and optical densities measured in a Spectronic 601 (trial 1) Due to technical difficulties in trial 2, tubes were refrigerated for 24 h, then optical densities measured in the Shimadzu at 625 nm.

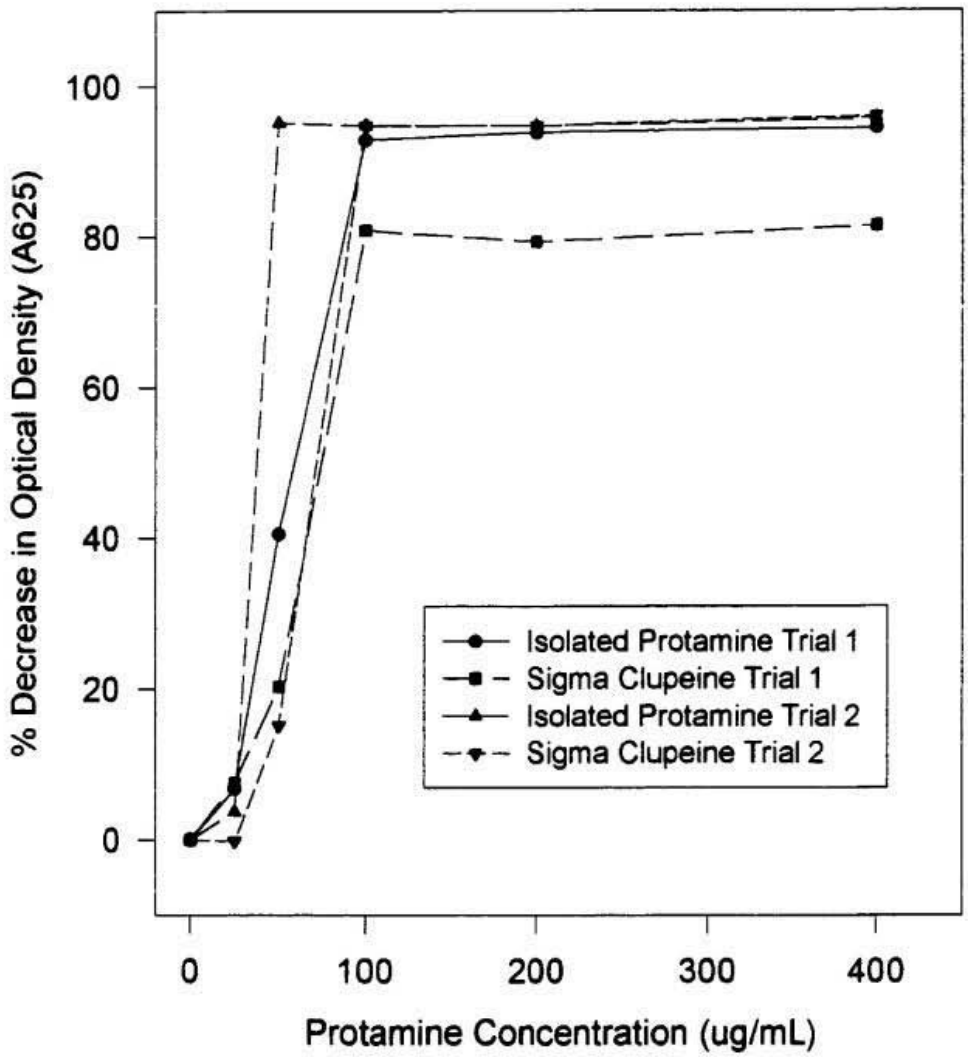


Figure 26: Effect of different concentrations of isolated protamine and Sigma clupeine on the growth of *Salmonella typhimurium*.
A standardized inoculum of *S. typhimurium* was prepared and 0.1 mL aliquots used to inoculate 2.4 mL of MHB containing different concentrations of isolated protamine or clupeine (Sigma) in 16x125 mm test tubes. The initial level of bacteria was 4.85×10^5 CFU/mL for trial 1 and 1.94×10^5 CFU/mL for trial 2. Tubes were incubated at 37°C for 24 h and optical densities measured in a Spectronic 601 (trial 1) and a Shimadzu (trial 2) spectrophotometer at 625 nm.

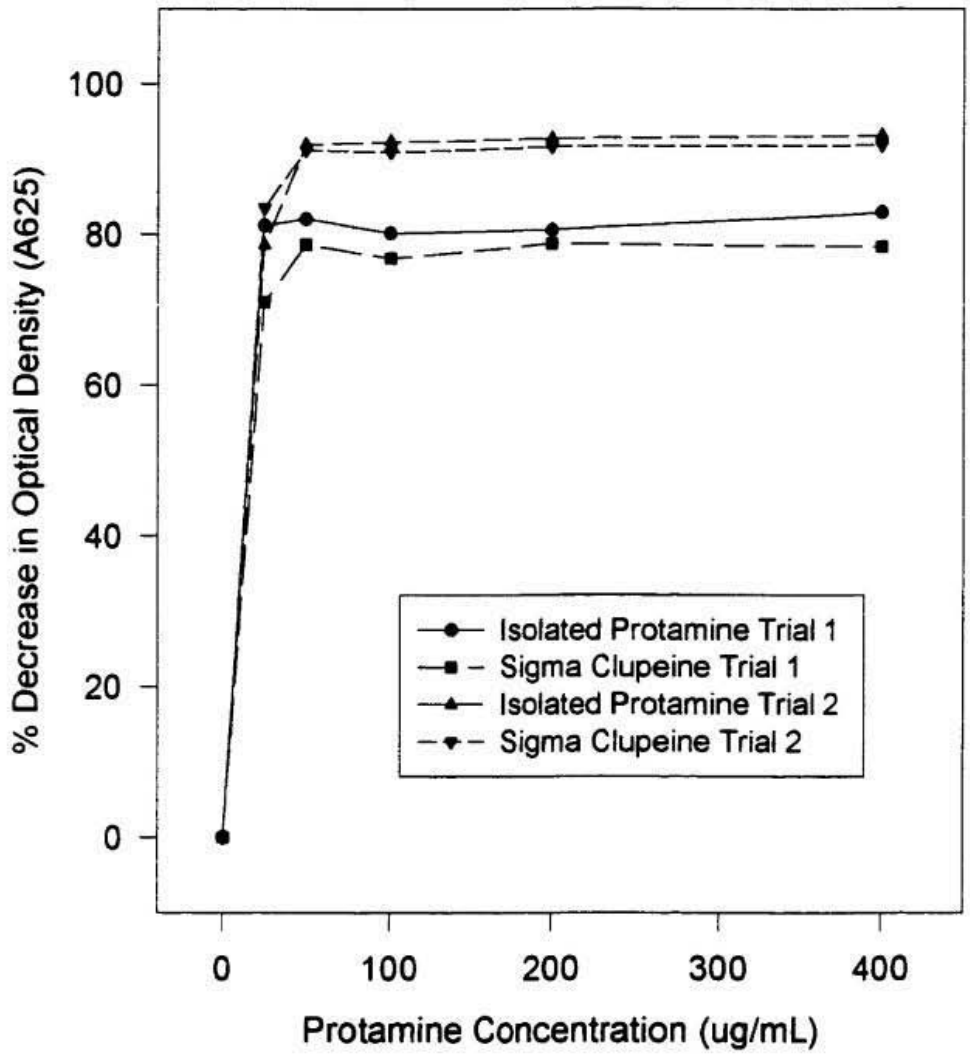


Figure 27: Effect of different concentrations of isolated protamine and Sigma clupeine on the growth of *Vibrio anguillarum*.
A standardized inoculum of *V. anguillarum* was prepared and 0.1 mL aliquots used to inoculate 2.4 mL of MHB (trial 1) and MHB + 100mM NaCl (trial 2) containing different concentrations of isolated protamine or clupeine (Sigma) in 16x125 mm test tubes. The initial level of bacteria was 7.50×10^4 CFU/mL for trial 1 and 2.30×10^5 CFU/mL for trial 2. Tubes were incubated at room temperature for 24 h (trial 1) and 48 h (trial 2) and optical densities measured in a Spectronic 601 (trial 1) and a Shimadzu (trial 2) spectrophotometer at 625 nm.

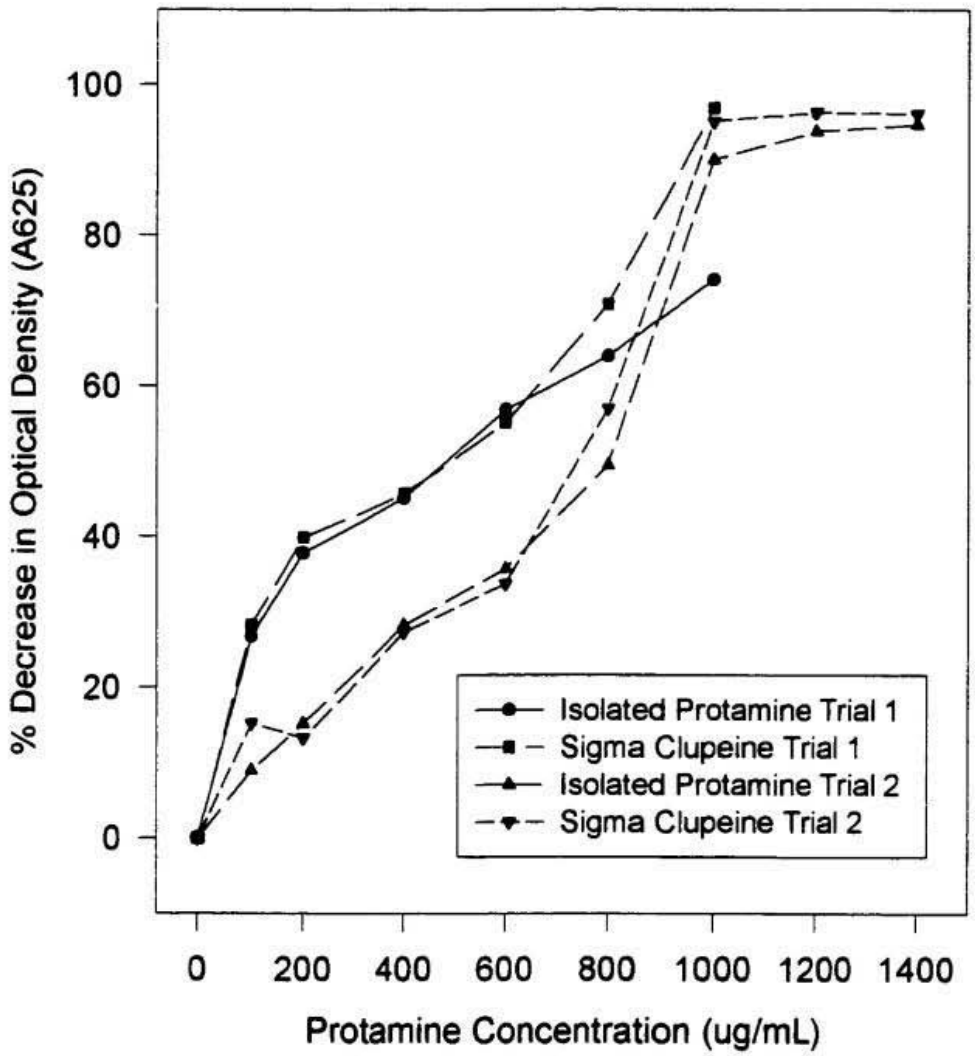


Figure 28: Effect of different concentrations of isolated protamine and Sigma clupeine on the growth of *Yersinia ruckerii*.
A standardized inoculum of *Y. ruckerii* was prepared and 0.1 mL aliquots used to inoculate 2.4 mL of MHB containing different concentrations of isolated protamine or clupeine (Sigma) in 16x125 mm test tubes. The initial level of bacteria was 4.60×10^5 CFU/mL for trial 1 and 4.85×10^5 CFU/mL for trial 2. Tubes were incubated at room temperature for 48 h and optical densities measured in a Shimadzu spectrophotometer at 625 nm.

For the second trial with the organisms, in addition to optical density measurements, the actual numbers of bacteria were determined via aerobic plate count (see Tables 4 - 14) . At protamine concentrations at, or above, the MIC, the maximum number of bacteria obtained via plate count was 10^5-10^6 CFU/mL, *A. salmonicida* (Table 6), which is approximately the initial number inoculated. However, bacterial numbers were often much lower than this, in the 100-1000 CFU/mL range, and were not even detected in some tubes such as *B. subtilis*, *P. fluorescens* and *V. anguillarum* (see Tables 3,10 and 12 respectively). The numbers in control tubes generally ranged from 10^8-10^9 CFU/mL.

Table 4: Number of *Bacillus subtilis* (CFU/mL) obtained by aerobic plate count after treatment with isolated protamine oil or Sigma clupeine.

	Isolated Protamine		Sigma Clupeine	
Protein Concentration (µg/mL)	Tube A	Tube B	Tube A	Tube B
0	1.18×10^7	1.07×10^7	1.32×10^7	1.08×10^7
25	1.53×10^5	1.57×10^5	9.45×10^5	1.29×10^5
50	1.57×10^5	4.68×10^3	2.60×10^4	8.60×10^2
100	0	20	30	0
200	10	0	0	0
400	0	0	0	LA

LA- laboratory accident

Table 5: Number of *Listeria monocytogenes* 4b (CFU/mL) obtained by aerobic plate count after treatment with isolated protamine oil or Sigma clupeine.

	Isolated Protamine				Sigma Clupeine			
Protein Conc. µg/mL	Trial 1		Trial 2		Trial 1		Trial 2	
	Tube A	Tube B	Tube A	Tube B	Tube A	Tube B	Tube A	Tube B
0	4.25×10^8	3.30×10^8	3.35×10^8	1.80×10^8	4.05×10^8	4.70×10^8	3.55×10^8	3.35×10^8
25	3.40×10^8	3.10×10^8	--	--	3.60×10^8	4.45×10^8	--	--
50	6.65×10^8	6.25×10^8	--	--	7.50×10^8	4.25×10^8	--	--
100	6.65×10^8	8.45×10^8	9.45×10^8	9.65×10^8	7.50×10^8	6.05×10^8	8.70×10^8	9.50×10^8
200	1.04×10^9	1.03×10^9	1.39×10^9	1.07×10^9	1.18×10^9	1.05×10^9	1.09×10^9	1.03×10^9
400	2.65×10^6	4.85×10^6	2.00×10^6	2.65×10^6	1.11×10^8	3.10×10^6	2.10×10^8	1.70×10^8
800	8.72×10^3	1.08×10^4	1.02×10^5	4.06×10^3	1.19×10^5	3.00×10^5	5.50×10^4	1.75×10^4

Table 6: Number of *Staphylococcus aureus* (CFU/mL) obtained by aerobic plate count after treatment with isolated protamine oil or Sigma clupeine.

	Isolated Protamine		Sigma Clupeine	
Protein Conc. µg/mL	Tube A	Tube B	Tube A	Tube B
0	5.45×10^6	6.35×10^6	7.90×10^6	6.40×10^6
25	1.02×10^6	6.73×10^5	9.06×10^5	9.49×10^5
50	3.29×10^5	4.40×10^5	8.94×10^5	3.21×10^5
100	1.27×10^5	1.19×10^5	1.67×10^5	1.87×10^5
200	3.30×10^4	6.40×10^4	1.14×10^5	1.54×10^5
400	1.80×10^4	3.25×10^4	7.50×10^3	1.15×10^4

Table 7: Number of *Aeromonas salmonicida* (CFU/mL) obtained by aerobic plate count after treatment with isolated protamine oil or Sigma clupeine.

	Isolated Protamine		Sigma Clupeine	
Protein Conc. µg/mL	Tube A	Tube B	Tube A	Tube B
0	2.82×10^9	3.99×10^9	2.33×10^9	4.12×10^9
100	1.20×10^9	1.86×10^9	5.40×10^8	7.95×10^8
200	6.16×10^8	4.59×10^8	7.22×10^8	5.97×10^8
400	3.57×10^8	2.93×10^8	3.56×10^8	4.36×10^8
600	5.19×10^8	5.49×10^8	4.18×10^8	4.21×10^8
800	2.15×10^7	6.98×10^8	2.63×10^7	1.63×10^9
1000	2.36×10^8	6.00×10^5	2.25×10^5	9.73×10^8
1200	1.77×10^8	7.68×10^8	2.10×10^5	4.85×10^5

Table 8: Number of *Aeromonas sobria* (CFU/mL) obtained by aerobic plate count after treatment with isolated protamine oil or Sigma clupeine.

	Isolated Protamine		Sigma Clupeine	
Protein Conc. µg/mL	Tube A	Tube B	Tube A	Tube B
0	1.93×10^9	1.27×10^9	1.30×10^9	1.43×10^9
50	1.62×10^9	1.10×10^9	1.34×10^9	9.55×10^8
100	1.55×10^8	2.60×10^8	9.55×10^8	9.90×10^8
200	5.50×10^5	1.50×10^6	3.40×10^6	5.00×10^6
400	2.32×10^5	1.94×10^5	5.60×10^5	8.53×10^5
600	3.75×10^4	4.95×10^4	1.35×10^5	9.75×10^4
800	8.52×10^3	9.19×10^3	4.00×10^4	3.80×10^4
1000	1.90×10^3	1.70×10^3	4.30×10^3	5.17×10^4

Table 9: Number of *Escherichia coli* (CFU/mL) obtained by aerobic plate count after treatment with isolated protamine oil or Sigma clupeine.

	Isolated Protamine		Sigma Clupeine	
Protein Conc. µg/mL	Tube A	Tube B	Tube A	Tube B
0	8.40×10^{6}	7.70×10^{6}	6.70×10^{6}	7.35×10^{6}
25	5.91×10^{5}	2.62×10^{5}	2.40×10^{6}	5.65×10^{6}
50	1.80×10^{4}	7.80×10^{4}	8.70×10^{4}	3.85×10^{4}
100	1.15×10^{4}	1.50×10^{4}	8.59×10^{3}	7.55×10^{4}
200	1.14×10^{4}	7.30×10^{3}	5.96×10^{3}	4.51×10^{3}
400	9.80×10^{2}	1.18×10^{3}	3.73×10^{3}	4.22×10^{3}

Table 10: Number of *Pseudomonas aeruginosa* (CFU/mL) obtained by aerobic plate count after treatment with isolated protamine oil or Sigma clupeine.

	Isolated Protamine		Sigma Clupeine	
Protein Conc. µg/mL	Tube A	Tube B	Tube A	Tube B
0	1.63×10^{9}	1.82×10^{9}	1.95×10^{9}	2.12×10^{9}
25	1.42×10^{9}	2.18×10^{9}	1.86×10^{9}	1.90×10^{9}
50	5.15×10^{6}	6.20×10^{6}	1.67×10^{9}	1.77×10^{9}
100	9.50×10^{7}	1.25×10^{4}	1.50×10^{7}	5.00×10^{7}
200	5.00×10^{2}	1.00×10^{3}	3.00×10^{6}	1.0×10^{3}
400	3.39×10^{3}	5.00×10^{2}	1.0×10^{3}	1.0×10^{3}

Table 11: Number of *Pseudomonas fluorescens* obtained by aerobic plate count after treatment with isolated protamine oil or Sigma clupeine.

	Isolated Protamine		Sigma Clupeine	
Protein Conc. µg/mL	Tube A	Tube B	Tube A	Tube B
0	1.01×10^{9}	3.10×10^{8}	1.50×10^{9}	1.10×10^{9}
25	1.55×10^{9}	1.70×10^{9}	9.80×10^{8}	8.80×10^{8}
50	9.50×10^{7}	2.20×10^{8}	6.80×10^{8}	7.20×10^{8}
100	3.30×10^{7}	4.30×10^{7}	1.50×10^{8}	1.70×10^{8}
200	9.70×10^{3}	180	1.20×10^{4}	3.60×10^{4}
400	0	0	1.93×10^{4}	0

Table 12: Number of *Salmonella typhimurium* (CFU/mL) obtained by aerobic plate count after treatment with isolated protamine oil or Sigma clupeine.

	Isolated Protamine		Sigma Clupeine	
Protein Conc. µg/mL	Tube A	Tube B	Tube A	Tube B
0	1.01×10^9	1.23×10^9	1.22×10^9	9.35×10^8
25	6.4×10^8	8.10×10^8	6.15×10^8	8.40×10^8
50	5.43×10^5	1.69×10^5	3.55×10^8	3.35×10^8
100	9.80×10^4	1.28×10^4	7.40×10^4	1.29×10^5
200	5.45×10^4	2.07×10^5	3.30×10^4	1.31×10^5
400	6.80×10^2	2.50×10^4	1.20×10^4	1.35×10^4

Table 13: Number of *Vibrio anguillarum* (CFU/mL) obtained by aerobic plate count after treatment with isolated protamine oil or Sigma clupeine.

	Isolated Protamine		Sigma Clupeine	
Protein Conc. µg/mL	Tube A	Tube B	Tube A	Tube B
0	3.20×10^8	3.55×10^8	8.05×10^8	9.45×10^8
25	6.54×10^5	4.62×10^5	1.04×10^4	1.35×10^5
50	35	1.70×10^4	4.89×10^3	3.18×10^3
100	9.50×10^3	0	0	145
200	515	0	10	0
400	145	0	0	0

Table 14: Number of *Yersinia ruckerii* (CFU/mL) obtained by aerobic plate count after treatment with isolated protamine oil or Sigma clupeine.

	Isolated Protamine		Sigma Clupeine	
Protein Conc. µg/mL	Tube A	Tube B	Tube A	Tube B
0	1.97×10^9	1.75×10^9	2.77×10^9	2.02×10^9
100	9.25×10^8	1.05×10^9	1.31×10^9	1.50×10^9
200	7.30×10^8	8.15×10^8	1.02×10^9	9.25×10^8
400	2.95×10^8	3.10×10^8	3.50×10^8	4.35×10^8
600	1.78×10^8	1.07×10^8	1.75×10^8	1.83×10^8
800	1.07×10^8	1.01×10^8	6.79×10^7	8.10×10^7
1000	3.26×10^8	4.51×10^8	8.83×10^5	5.60×10^5
1200	7.83×10^5	1.18×10^8	1.01×10^5	1.71×10^5
1400	5.99×10^5	2.03×10^5	7.92×10^3	2.59×10^4

4. DISCUSSION

4.1 Absorption Spectra

The isolated and commercial protamines each had only one major peak at approximately 210 nm. This represents the absorbance due to peptide bonds. The lack of absorbance peaks in the ultraviolet region above this reflects the absence of aromatic acids. This pattern is characteristic of most fish protamines because they most often lack aromatic acids and thus the only absorption peak is that of the peptide bond (Ando *et al.*, 1973).

4.2 Gel Electrophoresis

4.2.1 SDS-PAGE

Electrophoresis of protamines extracted from the first batch of gonads resulted in a triplet and a broad band whereas for protamine oil, batch 2, there was 1 band. This suggests that the protamine oil is made up of fewer components than the protamines extracted from the first batch. This is quite reasonable because the first batch of gonads were mature but of uncertain stage in spermatozoan development. Thus there could be modified or immature protamines present, or possibly histone remnants. Sample overload or degradation products could also cause a smearing effect. However, sample overload is a rather unlikely explanation because there was a higher amount of protamine loaded in lane 6 and there was very little smearing. Salmine from Sigma also gives a very broad band. This commercial preparation could contain protamines extracted from a number of different salmon gonads or even from different species which could have slightly different protamines present. There

was no observable band for clupeine from Sigma even though there was a band for Sigma salmine. This result is rather confusing given that salmine and clupeine are similar in amino acid composition and molecular weight. Perhaps the dye does not bind to clupeine for some reason or it was more loosely bound and therefore lost during de-staining. The molecular weights calculated from the standard curve for the isolated protamines and salmine from Sigma were higher than the molecular weights calculated from the published sequences (4000-4300 Da) for clupeine or salmine (Ando *et al.*, 1973). These molecular weight deviations are probably due to abberations in electrophoretic mobility owing to the low molecular weight and highly basic nature of these proteins.

4.2.2 PAGE

Every tube gel but two had a single band observable after electrophoresis. These were fractions A and B from the ion exchange chromatography of protamine isolated from the first batch of gonads. For peak B, the protamine was in such a dilute solution that there was <2 μg loaded unto the gel. This may not have been enough to stain in the gel. There was no protein detected in fraction A by Lowry protein estimation, Thus, the protamine in fraction A must have been even more dilute than fraction B. This will be discussed further in the section on chromatography. The other gels, whether from crude or fractionated protamines, had a single band. Protamines are often heterogeneous (Chevaillier, 1983) thus one might expect more than one band. However, the fractionated components, of the isolated protamines and clupeine

(Sigma), are so similar in length and amino acid sequence that they may not be easily resolved on polyacrylamide gels. Okamoto and colleagues (1993), using the same electrophoresis method, found only one band for clupeine. Scombrine II which is a fractionated component of scombrine γ, a protamine from spotted mackerel, also gave only 1 band even though scombrine I, a minor fraction, had 3 bands. Mugiline β, protamine from mullet, also gave only a single band using the same electrophoresis procedure (Reisfeld *et al.* ,1962), despite the fact that it is composed of 7 components (Okamoto *et al.*, 1987). Thus the appearance of a single band, even in heterogeneous whole protamine, is not unusual.

4.3 Ion Exchange Chromatography

The 8 peaks observed after the switch to elution buffer, in the ion exchange chromatography of protamine isolated from the first batch of gonads, reinforces the speculation that there are multiple components present as suggested by the SDS-PAGE results for this protamine extract described earlier. The chromatograms of commercial clupeine and protamine oil have 3 major peaks which is in agreement with results obtained by the authors who originated this fractionation procedure (Ando and Watanabe, 1969). The peak (A) found prior to the switch to elution buffer for the isolated protamine, batch 1, and the unlabelled early peak for protamine oil, occur so soon after protamine addition that they probably represent material that did not bind to the column. This could simply be the result of sample overload, that is adding more protamine than could bind to the ion exchange resin with the extra material passing right

through. Alternatively, the early peaks could represent a small amount of contaminating protein from the gonad which would probably be acidic rather than basic and hence would not bind to the resin. However, as previously described, protein was not detected in the A peak, for the first batch of protamine, via Lowry protein determination, nor did it give a band after electrophoresis and staining. Thus, the protein concentration must be very low.

4.4 Amino Acid Composition

The amino acid compositions of the fractionated components of protamine oil, MA, MB and MC, and Sigma clupeine, SA, SB and SC, correspond well with the published sequences of clupeine Y-II, Y-I and Z respectively (Ando *et al.*, 1973).

4.5 Electrospray Mass Spectra

The published amino acid sequences of the fractionated components of clupeine (Ando *et al.*, 1973) and the molecular weight (MW) of each component calculated from these sequences are summarized in Table 15.

Table 15: The amino acid sequences of clupeine Y-I, Y-II and Z and the molecular weight of each component as calculated from these sequences.

Fraction	Amino Acid Sequence	MW (Da)
Clupeine Y-I	H-Ala-$(Arg)_4$-$(Ser)_3$-Arg-Pro-Ile-$(Arg)_4$-Pro-$(Arg)_3$-$(Thr)_2$-$(Arg)_4$-Ala-Gly-$(Arg)_4$-OH	4111.82
Clupeine Y-II	H-Pro-$(Arg)_3$-Thr-$(Arg)_2$-Ala-Ser-Arg-Pro-Val-$(Arg)_4$-Pro-$(Arg)_2$-Val-Ser-$(Arg)_4$-Ala-$(Arg)_4$-OH	4048.80
Clupeine Z	H-Ala-$(Arg)_4$-Ser-$(Arg)_2$-Ala-Ser-Arg-Pro-Val-$(Arg)_4$-Pro-$(Arg)_2$-Val-Ser-$(Arg)_4$-Ala-$(Arg)_4$-OH	4164.93

Sequence data of Ando *et al.*, 1973

Therefore, from the data obtained by amino acid analysis, the predicted molecular weight of fractions MA and SA would be 4048.80, fractions MB and SB would be 4111.82 and MC and SC would be 4164.93. However, the ES-MS spectra showed heterogeneity in each of these fractions and a number of peaks of different molecular weights were found after maximum entropy processing. The minor peaks observed probably represent either background noise or minute quantities of cross contaminants from other fractions. The intermediate and major peaks will be discussed further in the following paragraphs.

Fraction MA does not have any intermediate intensity peaks however SA has peaks at 4265, 4360 and 4460 Da. There are major peaks at 4360 and 4460 Da. in fraction SC, thus their presence in SA probably results from peak overlap during chromatography. That is, the separation of the 3 components into 3 peaks was not perfect and each could contain amounts of the other 2 components. The peak at 4265 Da. is 216.20 Da. heavier than the predicted

weight of 4048.80. This could be explained by the addition of a threonine residue, a valine residue, and an hydroxyl group to the sequence for clupeine Y-II shown above .

For fractions MB and SB, the moderate intensity peak at 4115 Da. corresponds to the predicted molecular weight of 4111.82 Da. However one would expect a peak of higher intensity at this molecular weight given that it is the molecular weight according to the published sequence for this clupeine fraction. Both MB and SB also have a moderate intensity peak at 4505 Da. This is a 393.18 Da. increase in molecular weight over that predicted. The addition of 2 proline residues, a threonine residue and a phosphate group to the sequence for clupeine Y-I shown above could account for the increased weight. MB has a peak at 4200 Da. which is 88.18 Da. higher than the predicted weight. This could be explained by the presence of another serine residue. Finally SB has a peak at 4440 Da. which is 328.18 Da. higher than that predicted. The addition of 2 isoleucine residues and 1 threonine residue could account for this increase.

MC and SC have 1 intermediate peak in common, 4150 Da. This peak is also present as a major peak in fractions MA and SA and therefore its presence in the MC and SC fractions is probably due to peak overlap as previously described. This peak is 14.93 Da. lower than the predicted molecular weight of 4164.93 Da. Therefore it could also represent clupeine Z, as shown above, minus a nitrogen atom. The 4310 Da. intermediate peak in MC which is present as a major peak in MB can also be explained as peak overlap. MC has 2 other intermediate peaks, at 4205 and 4560, which differ from the predicted molecular weight of 4164.93 Da. by 40.08 and 395.08 Da. respectively. The 40.08 Da.

increase could be explained by the addition of a sodium ion and an hydroxyl group to the sequence for clupeine Z. The addition of 3 valine residues and either a proline residue or a sulfate group would account for the 395.08 Da. increase. SC also has 3 other peaks. The 4250 Da. peak is 85.07 Da. heavier than predicted which could be the result of an additional alanine residue or sodium acetate from the column buffer. There is a peak at 4400 Da. which is 235.08 Da. heavier than predicted. This could be explained by the addition of an arginine and an alanine residue or by an alanine residue, a serine residue, and sodium acetate. The peak at 4550 Da. represents an increase of 385.08 Da. and this could be the addition of 2 valine residues, a serine residue and a sulfate group.

Fractions MA and SA have the same 3 major peaks, 4150, 4245 and 4345 Da. which are 101.2, 196.2 and 296.2 Da. respectively higher than the predicted molecular weight. An additional threonine residue could account for the 101.2 Da. increase. A threonine residue and a phosphate group could explain the 196.2 Da. increase and a threonine residue, valine residue and a phosphate group could account for the 296.2 Da. increase.

Fractions MB and SB have 3 major peaks in common, 4210, 4310, and 4405 Da. which are 98.18, 198.18 and 293.18 Da. higher respectively in molecular weight than the predicted 4111.82 Da. The 98.18 Da. difference could be either an additional proline residue or a phosphate group. An additional threonine residue and a phosphate group could explain the 198.18 Da. increase. The 293.18 Da. difference could be an additional threonine residue, proline residue and a phosphate group. MB also has a major peak at

4300 Da. which is 188.18 higher than expected. The addition of an alanine residue and an isoleucine residue could explain this increased weight. SB also has major peaks at 4150, 4245 and 4345 Da. which are the same three major peaks found in SA. These are probably the result of peak overlap as previously described.

MC and SC have two major peaks in common, 4360 and 4460 Da. which are higher in molecular weight than the predicted 4164.93 Da. by 195.07 and 295.07 Da. respectively. The 195.07 Da. can be explained by an additional valine residue and a phosphate group and the 295.07 Da. by 2 valine residues and a phosphate group. MC also has 2 other major peaks at 4265 and 4450 Da. The first of these can be explained by an additional valine residue and the other by a serine residue, valine residue and a phosphate group. SC has 2 other major peaks, 4210 and 4310 Da., which are 2 of the major peaks found in SB. The presence of these in SC can also be ascribed to peak overlap.

In summary, peaks corresponding to the predicted molecular weights of fractions MA, MB, MC, SA, SB and SC were either absent or present as intermediate or minor intensity peaks. There were a number of other peaks which were apparently longer than the predicted sequence by 1-4 amino acids or whose sequence was modified by the addition of sulfate, phosphate or hydroxyl groups, or by sodium or acetate ions. The additional groups probably represent residual impurities from the extraction procedure and the ions probably came from the buffers used in ion exchange chromatography. These would simply represent modified forms of the original sequence. However, the presence of extra amino acids suggests that the fractionated protamines used in this study

are longer than the published amino acid sequences. The fact that the fractions obtained from isolated protamine and commercial clupeine from Sigma are very similar suggests that the original sequences of the fractions may be incomplete or that microheterogeneity exists within each fraction. The original sequences of clupeine Y-I, Y-II and Z from *Clupea harengus* were determined in the early 1970's (Ando *et al.*, 1973) and have apparently not been altered since that time. Perhaps the high sensitivity of ES-MS methodology has uncovered previously unknown protamine components or amino acids that had been missed in the original sequencing. Difficulty exists in purifying, determining the amino acid composition, and sequencing protamines. Purification via ion exchange chromatography is difficult because the components are so similar in size and amino acid composition. Amino acid analysis often yields incomplete information due to the disproportionately high arginine content. Serine and threonine residues are also partially destroyed during hydrolysis. Sequencing via automated Edman degradation is often problematic because the repetitive yield of the phenylthiohydantoin derivative of arginine decreases when the arginine residues are consecutive (Schindler *et al.*, 1991). Sequencing of each molecular weight peak using modern methodology in conjunction with ES-MS would be required to settle this dilemma, however that is beyond the scope of this master's thesis

4.6 Antimicrobial Activity

With clupeine from Sigma, trial 1, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhimurium* gave very unusual growth

inhibition patterns. For the first three organisms, there was an initial decrease in optical density of around 90%, however this dropped to 76-84% with increased protamine concentration. With *S. typhimurium*, the maximum was an 80% decrease in OD which, once reached, remained constant despite increasing protamine concentrations. One possible explanation for these odd patterns could be the presence of a growth promoting factor in the commercial preparation which would counteract growth inhibition when present in sufficient quantity. Braekkan and Boge (1964) found that a raw extract of herring gonads contained both vitamin B_6, which stimulated microbial growth, and protamine which inhibited microbial growth. However, they found that microbial growth was stimulated in the presence of low concentrations of the extract and inhibited in higher concentrations which is the opposite of the patterns described above. However, this does not rule out the possibility that there was a contaminant introduced into the protamine solution or present on the glassware which caused the growth promotion. Alternatively, the clupeine may have caused autolysis of some bacteria which in turn would provide a nutrient source for a subset of resistant bacteria. The growth inhibition pattern of *Pseudomonas fluorescens* is somewhat similar to the above in that the lowest optical density was obtained at 200 μg/mL, which is the MIC for the other trial, but the OD increases at 400 μg/mL. This turbidity was determined, by plate count, to be the result of bacterial growth and not precipitation of media components. This could be the result of the same reasons given above. These anomalous results will therefore be disregarded and the MIC's obtained for isolated protamine and Sigma

clupeine, trial 2, will be used for the rest of the discussion. For *Aeromonas sobria*, in trial 1 the MIC was >400 μg/mL for both isolated and Sigma protamines whereas in the second trial the MIC's were both 400 μg/mL. However, in trial 1, one of the duplicate tubes for protamine oil had a MIC of 400 μg/mL and one of the duplicates for Sigma clupeine had an OD of 0.143, instead of the required 0.1. From this it appears that these MIC's were also very close to 400 μg/mL.

Thus the MIC's for *S. aureus*, *B. subtilis* and *Listeria monocytogenes*, the three Gram-positive bacteria, were 25, 50 and 800 μg/mL respectively. The following results were obtained for the Gram-negative bacteria: 25-50 μg/mL for *E. coli* and *Vibrio anguillarum*; 50-100 μg/mL for *S. typhimurium*; 100-200 μg/mL for *Pseudomonas aeruginosa*; 200 μg/mL for *P. fluorescens*; ≥400 μg/mL for *A. sobria*; 800-1000 μg/mL for *A. salmonicida* and ≥ 1000 μg/mL for *Yersinia ruckerii*. Therefore both Gram-positive and Gram-negative bacteria exhibited varying degrees of sensitivity to protamines as determined by optical density measures. The responses to isolated protamine oil or Sigma clupeine were very similar.

Protamine also exhibited varying degrees of bactericidal activity. According to plate counts of trial 2, the numbers of bacteria decreased below the initial inoculum level of approximately $4x10^5$ CFU/mL in most of the organisms assayed. In some instances, the numbers of bacteria decreased below the level of detection, 10 CFU/mL. Again, bactericidal activity did not appear to be

related to the Gram-reaction of the bacterium because numbers varied from about 10^5 down to zero in species of both Gram-negative and Gram-positive organisms.

Therefore, according to the methods used in this thesis, both Gram-positive and Gram-negative bacteria were sensitive to the protamines isolated from herring and the commercial protamines from Sigma. The following table is a comparison of the MIC results obtained from this study with those of other authors.

Table 16: The minimum inhibitory concentrations determined, from different studies, for protamines against various bacterial species.

	Minimum Inhibitory Concentration (μg/mL)				
Organism	This study (a)	Brock, 1958 (b)	Islam *et al.*, 1984 (c)	Johansen *et al.*, 1995 (d)	Yanagimoto *et al.*, 1992 (e)
B. subtilis	50	8	125-200[f]		250-275
L. monocytogenes	800			1000	
S. aureus	25	64-125		500	>500
A. salmonicida	800-1000			4000	
A. sobria	≥ 400			>4000	
E. coli	25-50	>1000	> 500		> 500
P. aeruginosa	100-200	32			
P. fluorescens	200		> 500	3000	> 500
S. typhimurium	50-100				
V. anguillarum	25-50			1000	
Y. ruckerii	≥ 1000				

(a) MIC determined for clupeines using OD readings and MHB
(b) determined visually for salmine in BHI
(c) determined for clupeine using agar dilution method
(d) determined for salmine (Sigma) in TSB via impedimetry
(e) determined for salmine via agar dilution
f - varied with strain

The MIC's obtained in this study for the Gram-positive organisms are either slightly lower or within the range of values obtained by other authors. However, in most case , the MIC's obtained for the Gram-negative organisms are much lower than those quoted by other authors. Differences in the strains of bacteria or methodology used might account for these results. Lower MIC values are generally found with broth dilution protocols, however there has been difficulty in using this method due to precipitation when protamine is added to the broth. This made the determination of inhibition via visual or spectrophotometric readings impossible. Therefore growth inhibition in broth assays was usually determined via the laborious and time-consuming method of serial dilution and plate count. Protamine does not precipitate in Mueller-Hinton broth, which is the media recommended by the NCCLS for antimicrobial susceptibility testing in broth (NCCLS, 1993). Thus, inhibition could be determined via spectrophotometry or even via visual comparison of the experimental and control tubes. The greater antimicrobial activity in this study could be attributable then, to the lack of precipitation of protamine with media components. Without the precipitation, there is probably a greater amount of protamine available to interact with the bacteria causing growth inhibition or cell death.

Conclusions

The protamines isolated from local herring and commercial clupeine from Sigma have very similar properties. The absorption spectrum for each shows only 1 peak. The ion exchange chromatography of protamine oil and Sigma clupeine both had 3 peaks. The chromatogram for the first batch of isolated protamine had 9 peaks, but this is probably due to different stages of development of the sperm. In general, both the whole and fractionated protamine components show one band on PAGE. The result on SDS-PAGE are more variable. The amino acid compositions of the fractionated components are similar and correspond to the predicted compositions obtained from the published sequences. The ES-MS spectra contain many series of peaks of multiply charged ions indicating that a mixture of proteins exist. The mock spectra generated by the computer are very similar to the original scans which indicates that these peaks actually exist and the proteins are present. The maximum entropy processing determined the molecular weights of each of the protamine components. Each protamine fraction was made up of many components that were often higher in molecular weight than would be predicted from the published amino acid sequence. Some of these peaks were explained as overlap resulting from imperfect peak separation during ion exchange chromatography. Others peaks probably resulted from modifications to the original sequence by residual impurities from the extraction procedure or ions contained in the buffers used. Some peaks, however, appeared to have additional amino acids. Therefore the published amino acid sequence may not be complete or additional protamine components could be present in each

fraction. Both protamine oil and Sigma clupeine exhibited antimicrobial activity against Gram-positive and Gram-negative bacteria. The MIC's found for the Gram-negative bacteria were much lower than those of other authors using the same bacterial species. It appears that the method used greatly influences the outcome of antimicrobial susceptibility tests, particularly where Gram-negative bacteria are concerned. Gram-negatives comprise a very important group of bacteria with respect to food because many of them are responsible for foodborne illness. Therefore, a standardized method of determining the effectiveness of protamines against food-borne organisms is necessary if these proteins are going to be used as food preservatives.

REFERENCES

Ando, T. and Watanabe, S. 1969. A new method for fractionation of protamines and the amino acid sequences of salmine and three components of iridine. Int. J. Prot. Res. **1**: 221-224.

Ando, T., Yamaski, M. and Suzuki, K. 1973. Molecular Biology, Biochemistry and Biophysics 12. Protamines. Isolation, Characterization, Structure and Function. Springer-Verlag, New York, Heidelberg, Berlin. 116 pp.

Antohi, S. and Popescu, A. 1979a. Lethal effect of protamine and histone on competent *Bacillus subtilis* cells. Inhibition of genetic transformation by protamine in sublethal concentration. Molec. Gen. Genet. **170**: 345-349.

Antohi, S. and Popescu, A. 1979b. Protamine and polyarginine bacteriolysis. Similarities in its mechanism with chromatin DNA picnosis. Z. Naturfosch. **34c**: 1144-1150.

Aspedon, A. and Groisman, E. A. 1996. The antibacterial action of protamine: evidence for disruption of cytoplasmic membrane potential in *Salmonella typhimurium*. Microbiology **142**: 3389-3397.

Balhorn, R. 1989. Mammalian protamines: structure and molecular interactions. *In* Molecular Biology of Chromosome Function. *Edited by* K. W. Adolph. Springer-Verlag, New York, pp. 360-395.

Balhorn, R. 1982. A model for the structure of chromatin in mammalian sperm. J. Cell Biol. **93**: 298-305.

Banoub, J., Gentil, E. and Kiceniuk, J. 1995. Characterization of triazine herbicides by electrospray and tandem mass spectrometry. Intern. J. Environ. Anal. Chem. **61**: 11-26.

Bazett-Jones, D. P. and Ottensmeyer, F. P. 1979. A model for the structure of nucleoprotamine. J. Ultrastruct. Res. **67**: 255-266.

Bellvé, A. R. 1979. *In* Oxford Reviews of Reproductive Biology. *Edited by* C. A. Finn. Clarendon Press, Oxford, 1, pp. 159-261. Quoted by Chevaillier, P. 1983. Some aspects of chromatin structure in sperm nuclei. *In* The Sperm Cell. *Edited by* J. André. Martinus Nijhoff, The Hague, pp. 179-196.

Bloch, D. P. 1969. A catalog of sperm histones. Genetics Suppl. **61**: 93-111.

Bloch, D. P. 1976. Histones of sperm. *In* Handbook of Genetics. Vol. 5 Molecular Genetics. *Edited by* R. C. King. Plenum Press, New York. pp. 139-167.

Braekkan, O.R. and Boge, G. 1964. Growth inhibitory effect of extracts from milt (testis) of different fishes and of pure protamines on microorganisms. Fiskeridrektoratets skrifter **4**: 1-22.

Brock, T. D. 1958. The effect of salmine on bacteria. Can. J. Microbiol. **4**: 65-71.

Chapman, J. R. Mass spectrometry. Ionization methods and instrumentation. *In* Protein and Peptide Analysis by Mass Spectrometry. Methods in Molecular Biology , Volume 61. *Edited by* J. R. Chapman. Humana Press, Totowa, New Jersey, pp. 9-28.

Chargaff, E and Olson, K. B. 1937. Studies on the chemistry of blood coagulation VI. Studies on the action of heparin and other anticoagulants. The influence of protamine on the anticoagulant effect *in vivo*. J. Biol. Chem **122**: 153-167.

Chevaillier, P. 1983. Some aspects of chromatin structure in sperm nuclei. *In* The Sperm Cell. *Edited by* J. André. Martinus Nijhoff, The Hague, pp. 179-196.

Csaba, G, Acs, T., Horvath, C. and Kapa, E. 1960. Some new data concerning the biology of tumours. Brit. J. Cancer **14**: 367-375.

Daly, M. M., Mirsky, A. E. and Ris, H. 1951. The amino acid composition and some properties of histones. J. Gen. Physiol. **34**: 439-450.

Dixon, G. H., Aiken, J. M., Jankowski, J. M., McKenzie, D. I., Moir, R. and States, J. C. 1985. Organization and evolution of the protamine genes of salmonid fishes. *In* Chromosomal Proteins and Gene Expression. *Edited by* G. R. Reeck, G. H. Goodwin and P. Puigdomenech. Plenum Press, New York and London, pp. 287-314.

Ebert, G., Nishi, N. and Zolzer, U. 1992. Reverse micelles as a tool for elucidating the conformation of protamines in sperm-head nucleoproteins of some fish. Colloids Surfaces **67**:157-163.

Felix, K. 1960. Protamines. *In* Advances in Protein Chemistry. Vol. 15. *Edited by* C. B. Anfinsen, Jr., M. L. Anson, K. Bailey and J. T. Edsall. Academic Press, New York, London. pp. 1-56.

Feughelman, M., Langridge, R., Seeds, W. E., Stokes, A. R., Wilson, H. R., Hooper, C. W., Wilkins, M. H. F., Barclay, R. K. and Hamilton, L. D. 1955. Molecular structure of deoxyribose nucleic acid and nucleoprotein. Nature **175**: 834-838.

Garvie, W. H. H. 1965. The action of protamine derivatives and nitrogen mustard on the growth of the Walker 256 rat carcinoma. Brit. J. Cancer **19**: 519-526.

Gottschlich, G. M., Gravlee, G. P. and Georgitis, J. W. 1988. Adverse reactions to protamine sulfate during cardiac surgery in diabetic and non-diabetic patients. Ann. Allergy **61**: 277-281.

Hagedorn, H. C., Norman Jensen, B., Krarup, N. B. and Wodstrup, I. 1936. Protamine insulinate. J. Amer. Med. Assoc. **106**: 177-180.

Hecht, N. B. 1989. Mammalian protamines and their expression. *In* Histones and Other Basic Nuclear Proteins. *Edited by*: L. S. Hnilica, G. S. Stein and J. L. Stein. CRC Press, Inc. , Boca Raton, Florida. pp. 347-373.

Hirsch, J. G. 1958. Bactericidal action of histone. J. Exp. Med. **108**: 925-944.

Horrow, J. C. 1985. Protamine: a review of its toxicity. Anesth. Analg. **64**: 348-361.

Hughes, L. E. 1964. Treatment of malignant disease with protamine sulphate. Lancet **i**: 408-409.

Islam, N., Itakura, T. and Motohiro, T. 1984. Antibacterial spectra and minimum inhibitory concentration of clupeine and salmine. Bull. Jap. Soc. Sci. Fish. **50**: 1705-1708.

Islam, N., Motohiro, T. and Itakura, T. 1985a. Effects of pH, temperature, metal ions and organic matters on the bactericidal action of clupeine sulfate. Bull. Jap. Soc. Sci. Fish. **51**(5): 811-815.

Islam N., Motohiro, T. and Itakura, T. 1985b. Antibacterial action of fractionated components of clupeine sulfate and salmine sulfate. Bull. Japan. Soc. Sci. Fish. **51**: 995-999.

Islam, N., Motohiro, T. and Itakura, T. 1986a. Inhibitory effect of protamine on the growth from spores of two *Bacillus* species. Bull. Japan. Soc. Sci. Fish. **52**: 913-917.

Islam, N., Motohiro, T. and Itakura, T. 1986b. Combined effect of heat treatment and protamine on the growth and heat resistance of *Bacillus* spores. Bull. Japan. Soc. Sci. Fish. **52**: 919-922.

Islam, N., Oda, H. and Motohiro, T. 1987. Changes in the cell morphology and the release of soluble constituents from the washed cells of *Bacillus subtilis* by the action of protamine . Nippon Suisan Gakkaishi. **53**: 297-303.

Islam, N., Motohiro, T. and Kimura, T. 1987a. Antibacterial characteristics of fish protamines. VII. Interaction between clupeine sulfate and the cytoplasmic membrane of *Bacillus subtilis*. J. Gen. Appl, Microbiol. **33**: 481-487.

Islam, N., Motohiro, T. and Kimura, T. 1987b. Antibacterial characteristics of fish protamines. VIII. Effect of clupeine sulfate on the membrane ATPase activity and membrane structure of *Bacillus subtilis*. J. Gen. Appl. Microbiol. **33**: 489-496.

Jaques, L. B. 1973. Protamine - antagonist to heparin. C.M.A. Journal **108**: 1291-1297.

Johansen, C., Gill, T. and Gram, L. 1995. Antibacterial effect of protamine assayed by impedimetry. J. Appl. Bacteriol. **78**: 297-303.

Kamal, Md., Motohiro, T. and Itakura, T. 1986. Inhibitory effect of salmine sulfate on the growth of molds. Bull. Jap. Soc. Sci. Fish. **52**: 1061-1064.

Kamal, Md. and Motohiro, T. 1986. Effect of pH and metal ions on the fungicidal action of salmine sulfate. Bull. Jap. Soc. Sci. Fish. **52**: 1843-1846.

Kasinsky, H. E., Mann, M., Lemke, M. and Huang, S. Y. 1985. Diversity of sperm basic chromosomal proteins in the vertebrates: a phylogenetic point of view. *In*: Chromosomal Proteins and Gene Expression. *Edited by* G. R. Reeck, G. H. Goodwin and P. Puigdomènech, Plenum Press, New York. pp. 333-352.

Kleczkowski J. and Kleczkowski, A. 1956. Effects of clupeine and of its degradation products on a rhizobium bacteriophage, on its host bacterium and on the interaction between the two. J. Gen. Microbiol. **14**: 449-459.

Kossel, A. 1896a. Über die basischen Stoffe des Zellkerns. S. -B. Kgl. Preuss. Akad. Wiss. **18**: 403-408. Quoted in Ando, T., Yamaski, M. and Suzuki, K. 1973. Molecular Biology, Biochemistry and Biophysics 12. Protamines. Isolation, Characterization, Structure and Function. Springer-Verlag, New York, Heidelberg, Berlin. 116 pp.

Kossel, A. 1896b. Über die basischen Stoffe des Zellkerns. Z. Physiol. Chem. **22**: 176-187. Quoted by Ando, T., Yamaski, M. and Suzuki, K. 1973.

Molecular Biology, Biochemistry and Biophysics 12. Protamines. Isolation, Characterization, Structure and Function. Springer-Verlag, New York, Heidelberg, Berlin. 116 pp.

Kossel, A. 1928. The Protamines and Histones (Translated by W.V. Thorpe). Longmans, Green and Co., London-New York-Toronto.

Kouach, M., Jaquinod, M., Belaiche, D., Sautiere, P., van Dorsselaer, A., Chevaillier, P. and Briand, G. 1993. A corrected primary structure for dog-fish *Scylliorhinus caniculus* protamine Z3. Biochim. Biophys. Acta **1162**: 99-104.

Larsen, B. 1967. Increased permeability to albumin induced with protamine in modified gelatine membranes. Nature **215**: 641-642.

Leitch, G. J. and Tobias, J. M. 1963. Phospholipid-cholesterol membrane model: effects of calcium, potassium or protamine on membrane hydration, water permeability and electrical resistance. J. Cell. Comp. Physiol. **63**: 225-232.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265-275.

Lutton, A. 1964. Treatment of malignant disease with protamine sulphate. Lancet **i**: 768-769.

Luzzati, V. and Nicolaieff, A. 1963. The structure of nucleohistones and nucleoprotamines. J. Mol. Biol. **7**: 142-163.

Maier, W.-M., Nussbaum, G., Domenjoud, L., Klemm, U. and Engel, W. 1990. The lack of protamine 2 (P2) in boar and bull spermatozoa is due to mutations within the P2 gene. Nucl. Acid Res. **18**: 1249-1254.

Mann, M., Shen, S. and Fenn, J. B. Electrospray mass spectrometry. *In* Mass Spectrometry in the Biological Sciences: A Tutorial. *Edited by* M. L. Gross. Kluwer Academic Publishers, Norwell, MA., pp. 145-164.

Martin-Ponthieu, A., Wouters-Tyrou, D., Belaiche, D., Sautiere, P., Schindler, P. and Van Dorsselaer, A. 1991. Cuttlefish sperm protamines. 1. Amino acid sequences of two distinct variants. Eur. J. Biochem. **195**: 611-619.

Matsudomi, N., Tsujimoto, T., Kato, A. and Kobayashi, K. 1994. Emulsifying and bactericidal properties of a protamine-galactomannan conjugate prepared by dry heating. J. Food Sci. **59**: 428-431.

McClean, D. 1930. The influence of testicular extract on dermal permeability and the response to vaccine virus. J. Path. Bacteriol. **33**: 1045-1070.

McClean, D. 1931. Further observations on testicular extract and its effect upon tissue permeability. J. Path. Bacteriol. **34**: 459-470.

Miescher, F. 1874. Das protamin, eine neue organische base aus des Samenfäden des Rheinlachses. Ber. **7**: 376-379. Quoted by Ando, T., Yamaski, M. and Suzuki, K. 1973. Molecular Biology, Biochemistry and Biophysics 12. Protamines. Isolation, Characterization, Structure and Function. Springer-Verlag, New York, Heidelberg, Berlin. 116 pp.

Miller, B. F., Abrams, R., Dorfman, A. and Klein, M. 1942. Antibacterial properties of protamine and histone. Science **96**: 428-430.

Miyake, S. 1927. Die isoelektrischen Punkte der Protamine. Z. Physiol. Chem. **172**: 225-229. Quoted by Ando, T., Yamaski, M. and Suzuki, K. 1973. Molecular Biology, Biochemistry and Biophysics 12. Protamines. Isolation, Characterization, Structure and Function. Springer-Verlag, New York, Heidelberg, Berlin. 116 pp.

Muggleton, P. W., MacLaren, J. G. and Dyke, W. J. C. 1964. Effect of protamine sulphate on experimental tumours in mice. Lancet **i**: 409-410.

National Committee for Clinical Laboratory Standards. 1993. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically - Third Edition; Approved Standard. NCCLS document M7-A3. NCCLS, Villanova, Pennsylvania 19805.

O'Meara, R. A. Q and O'Halloran, M. J. 1963. Protamine derivatives in the treatment of advanced carcinoma of the breast. Lancet **ii**: 613-614.

Okamoto, Y., Muta, E. and Ota, S. 1987. Primary structures of M6 and M7 of mugiline β (*Mugil japonicus*). J. Biochem. **101**: 1017-1024.

Okamoto, Y., Kuno, K., Motohiro, T., Nishi, N., Muta, E. and Ota, S. 1992. Primary structures of sardaines Z1 and Z2, protamines isolated from striped bonito (*Sarda orientalis*). J. Biochem. **111**: 157-161.

Okamotoa, Y., Ogawa, K., Motohiro, T., Nishi, N., Muta, E. and Ota, S. 1993. Primary structure of scombrine γ, protamine isolated from spotted mackerel (*Scomber australasicus*). J. Biochem. **113**: 658-664.

Oliva, R. and Dixon, G. H. 1991. Vertebrate protamine genes and the histone-to-protamine replacement reaction. *In*: Progress in Nucleic Acid Research and Molecular Biology Volume 40. *Edited by*: W. E. Cohn and K. Moldave. Academic Press, Inc., San Diego, California. pp. 25-94.

Ottensmeyer, F. P., Whiting, R. F. and Korn, A. P. 1975. Three-dimensional structure of herring sperm protamine Y-I with the aid of dark field electron microscopy. Proc. Natl. Acad. Sci. USA **72**: 4953-4955.

Pirhonen, A., Linnala-Kankkunen, A. and Maenpaa, P. H. 1989. Comparison of partial amino acid sequences of two protamine 2 variants from stallion sperm. Structural evidence that the variants are products of different genes. FEBS Letters **244**: 199-202.

Reiner, L., DeBeer, E. J. and Green, M. 1942. Toxic effects of some basic proteins. Proc. Soc. Exp. Biol. And Med. **50**: 70-74.

Sautiere, P., Briand, G., Gusse, M. and Chevaillier, P. 1981. Primary structure of a protamine isolated from the sperm nuclei of the dog-fish *Scylliorhinus caniculus*. Eur. J. Biochem. **119**: 251-255.

Schindler, P., Bitsch, F., Klarskov, K., Roepstorff, P., Briand, G., Wouters-Tyrou, D., Sautiere, P. and Van Dorsselaer, A. 1991. Cuttlefish sperm protamines. 2. Mass spectrometry of protamines and related peptides. Eur. J. Biochem. **195**: 621-629.

Subirana, J. A. 1983. Nuclear proteins in spermatozoa and their interactions with DNA. *In*: The Sperm Cell. *Edited by* J. André, Martinus Nijhoff, The Hague. p. 197-213.

Taylor, S. and Folkman, J. 1982. Protamine is an inhibitor of angiogenesis. Nature **297**: 307-312.

Toniolo, C. 1980. Secondary structure prediction of fish protamines. Biochim. Biophys. Acta **624**: 420-427.

Uyttendaele, M. and Debevere, J. 1994. Evaluation of the antimicrobial activity of protamine. Food Microbiol. **11**: 417-427.

Warrant, R. W. and Kim, S.-H. 1978. α-Helix-double helix interaction shown in the structure of a protamine-transfer RNA complex and a nucleoprotamine model. Nature **271**: 130-135.

Yanagimoto, T., Tanaka, M. and Nagashima, Y. 1992. Changes in the antibacterial activity of salmine during its maillard reaction. Nippon Suisan Gakkaishi **58**: 2153-2158.

APPENDIX

A. Protein Extraction

Buffer 1
75mM NaCl containing 25mM NaEDTA (pH 8.0) and 0.5mM PMSF

-NaCl 4.4 g
-NaEDTA 9.3 g
-PMSF 0.087 g
-1 L distilled water

0.2M Sulfuric Acid
-10.66 mL of 36N H_2SO_4
-make to 1 L with distilled water

B. Lowry Protein Estimation

Stock Solutions

A: 2% sodium potassium tartrate solution *
B: 1% $CuSO_4 \bullet 5H_2O$ solution *
C: 0.2N NaOH solution *
D: 4% Na_2CO_3 solution *

* in distilled water

Assay Reagents

AB - 1 volume of A + 1 volume of B
CD - 1 volume of C + 1 volume of D
Copper Reagent - 50 mL CD + 1 mL AB
Phenol Reagent - commercial preparation supplied as 2N or 3N. Dilute appropriately in distilled water to obtain 1N solution.

Table A.1: Optical density obtained for BSA standards used to construct the standard curve for protamine batch 1.

Standard Protein (μg)	Absorbance (600 nm) Tube A	Tube B
0	0.001	0.002
20	0.271	0.256
40	0.442	0.466
60	0.533	0.536
80	0.732	0.734
100	0.918	0.918

Table A.2: Amount of protamine present in the combined protamine extract from batch 1.

Sample volume (μL)	Absorbance (600 nm)		Protein content (μg)		Average amount (μg)	Protamine concentration (mg/mL)
10	0.368	0.380	36.796	38.208	37.5	7.5
20	0.690	0.714	73.331	76.002	74.7	7.48

Sample calculation
37.5 μg/ 10 μL= 3.75 μg/μL x 2*= 7.5 μg/μL or 7.5 mg/mL

*Protamine was initially diluted in an equal amount of sterile distilled water.

Table A.3: Optical density obtained for BSA standards used to construct the standard curve for protamine batch 2.

Standard protein (μg)	Absorbance (600 nm) Tube A	Tube B
0	0.000	0.000
20	0.237	0.248
40	0.419	0.421
60	0.617	0.618
80	0.705	0.772
100	0.929	0.883

Table A.4: Amount of protamine present in the protamine extract from batch 2.

Sample	Volume (μL)	Absorbance (600 nm)		Protein content (μg)		Average	Protamine Concentration (mg/mL)
"Oil"	10	0.5594	0.5543	57.949	56.267	57.113	5.71
	20	0.7863	0.8148	83.165	86.325	84.745	4.24
Other	10	0.2871	0.2985	27.688	28.963	28.326	2.83
	20	0.4150	0.4022	41.903	40.479	41.191	2.06

Note: The concentrations obtained for the 10 and 20 μL samples were averaged to obtain 4.98 mg/mL for protamine oil and 2.45 mg/mL for other. The values for protein content were calculated automatically from the standard curve generated by the Shimadzu spectrophotometer.

C. Electrophoresis

SDS-PAGE (Schagger and von Jagow, 1987)

Stock solutions

A. Acrylamide Solution (49.5%T, 3%C)

Acrylamide 48 g
bis 1.5g

Dissolve in water to a final volume of 100 mL.

B. Gel Buffer

Tris Base 36.34g
SDS 0.30g

Dissolve in 60 mL water (gentle warming may be necessary). Adjust to pH 8.45 with concentrated HCl. Make up to final volume of 100 mL with water.

C. SDS Stock Solution , 20%

SDS 10.0g

Dissolve in 50 mL of water. Warm gently to completely dissolve.

D. Tris-HCl, 1M pH 6.8

Tris Base	12.1g

Dissolve in 80 mL water. Adjust to pH 6.8 with concentrated HCl. Make to 100 mL with water.

E. Sample Buffer

20% SDS	4.0 mL
Glycerol	2.4 mL
2-mercaptoethanol	0.4 mL
Bromophenol Blue*	2.0 mg
Tris-HCl, 1M, pH 6.8	1.0 mL

Make to final volume of 20.0 mL with water.
*Bromophenol blue was substituted for brilliant blue G (the suggested marker dye according to the protocol).

F. Ammonium Persulfate Solution

Prepare fresh daily by dissolving 100 mg ammonium persulfate in 1.0 mL of water.

G. Anode Buffer

Tris Base	121.1g

Dissolve in 1 L of water. Adjust to pH 8.9 with concentrated HCl. Make to final volume of 5 L with water.

H. Cathode Buffer

Tris Base	12.11g
Tricine	17.92g
SDS	1.0 g

Dissolve in 1 L of water. The pH should be approximately 8.2.

I. Fixative Solution

Methanol	50 mL
Acetic acid (glacial)	10 mL

Make to 100 mL with water.

J. Staining Solution

Coomassie brilliant blue R-250* 50 mg

Dissolve in 200 mL 10% acetic acid. Stir for 30 min. and filter. Stain may be used twice.

*Coomassie brilliant blue R-250 was substituted for the recommended brilliant blue G.

K. Destaining Solution

Acetic acid (glacial) 100 mL

Combine with 900 mL of water. Discard after one use.

Table A.5: Gel formulations for SDS-PAGE

	Separating Gel 16.5%T, 3%C	Spacer Gel 10% T, 3%C	Stacking Gel 4% T, 3%C
Acrylamide	10.0 mL	6.1 mL	1.0 mL
Gel Buffer	10.0 mL	10.0 mL	3.1 mL
Glycerol	3.2 mL	-	-
Water	6.8 mL	13.9 mL	8.4 mL

Table A.6: Relative mobilities and molecular weights of bands obtained after SDS-PAGE.

Sample	Lane	Distance migrated (cm)	Relative mobility (R_f)	Molecular weight (Da)
Tracking dye	all	9.1	1.0	NA
Myoglobin (fragment III)	1	8.9	0.98	2510
Glucagon	1	8.7	0.96	3480
Myoglobin (fragment II)	1	8.4	0.92	6210
Myoglobin (fragment I)	1	7.4	0.81	8160
Myoglobin (fragments I + III)	1	7.1	0.78	10600
Myoglobin (fragments I + II)	1	6.3	0.69	14440
Myoglobin (poly-peptide backbone)	1	5.8	0.64	16950
Protamine oil	2 & 6	8.0	0.88	~7100
Protamine (batch 1)	3	8.4	0.92	~6200
Protamine (batch 1)	3	7.8	0.86	~7600
Protamine (batch 1)	3	7.5	0.82	~9000
Protamine (batch 1)	3	7.4	0.81	~9200
Salmine (Sigma) *	5	7.6 (top) 8.1 (middle) 8.5 (bottom)	0.84 0.89 0.93	~8200 ~6800 ~5400

* This was a very broad band, so I measured from the top, middle and bottom of the band.

PAGE (Reisfeld *et al.*,1962)

Stock Solutions

A. N Potassium hydroxide	48 mL
Acetic acid (glacial)	17.2 mL
TEMED	4.0 mL
Water to make	100 mL
B. N Potassium hydroxide	48 mL
Acetic acid (glacial)	2.87 mL
TEMED	0.46 mL
Water to make	100 mL
C. Acrylamide	60 g
Methylene bis acrylamide	0.4 g
Water to make	100 mL
D. Acrylamide	10 g
Methylene bis acrylamide	2.5 g
Water to make	100 mL
E. Riboflavin	4.0 mg
Water to make	100 mL

Table A.7: Gel formulations for PAGE

	Small Pore Gel	Large Pore Gel
Stock solution A	6 mL	--
Stock solution B	--	1.5 mL
Stock solution C	12 mL	--
Stock solution D	--	3.0 mL
Stock solution E	--	1.875 mL
Urea	17.28 g	4.32 g
Water	18 mL	5.625 mL
APS*	12 mL	--
Total Volume	48	12 mL

*Ammonium persulphate solution
0.28 g ammonium persulphate in 100 mL water

Tray Buffer (pH 4.5)

β-alanine	31.2 g
Glacial acetic acid	8.0 mL
Water to make	1 L

Sample Solution

20% Sucrose	40 μL
0.005% Pyronine Y	25 μL
15 M Urea	75 μL
Sample	5-60 μL[A]
Water	0-55 μL[B]
Total Volume	200 μL

[A] - volume added depended on protamine concentration
[B] - appropriate amount of water added to bring volume up to 200 μL

D. Ion Exchange Chromatography

Stock solutions

0.2 M Acetic acid
0.2 M Sodium acetate

0.2 M Acetate buffer
91 mL of 0.2 M sodium acetate for every 9 mL of 0.2 M acetic acid

Equilibration Buffer - 0.05 M Acetate buffer containing 0.5 M NaCl

0.2 M Acetate buffer	250 mL
2.0 M NaCl	250 mL
Distilled water	500 mL

Elution Buffer - 0.05 M Acetate buffer containing 1.5 M NaCl

0.2 M Acetate buffer	250 mL
2.0 M NaCl	750 mL

E. Antimicrobial Assays

Table A.8: Preparation of different concentrations of protamine for the broth dilution assay.

Amount of Protein (μL)	Amount of Broth (mL)	Amount of Inoculum (mL)	Total Volume (mL)	Concentration of Protein (μg/mL)
0	2.4	0.1	2.5	0
12.5	2.3875	0.1	2.5	25
25	2.375	0.1	2.5	50
50	2.35	0.1	2.5	100
100	2.3	0.1	2.5	200
200	2.2	0.1	2.5	400
300	2.1	0.1	2.5	600
400	2.0	0.1	2.5	800
500	1.9	0.1	2.5	1000
600	1.8	0.1	2.5	1200
700	1.7	0.1	2.5	1400

Table A.9: Optical density (A625) readings obtained for *Aeromonas salmonicida* grown in the presence of different concentrations of isolated protamine and Sigma clupeine.

	Isolated Protamine				Sigma Clupeine			
Protein Conc. μg/mL	Trial 1		Trial 2		Trial 1		Trial 2	
0	1.363	1.332	1.350	1.458	1.422	1.291	1.410	1.262
100	1.209	1.151	1.103	1.101	1.300	1.307	1.191	1.145
200	1.044	1.015	0.996	0.952	1.085	1.090	1.037	0.981
400	0.834	0.821	0.760	0.767	0.912	0.924	0.865	0.827
600	0.765	0.721	0.622	0.664	0.743	0.729	0.669	0.678
800	0.529	0.113	0.086	0.068	0.473	0.118	0.103	0.206
1000	0.064	0.058	0.045	0.044	0.086	0.043	0.039	0.049
1200	0.067	0.053	0.041	0.041	0.031	0.040	0.037	0.036

Table A.10: Optical density (A625) readings obtained for *Pseudomonas aeruginosa* grown in the presence of different concentrations of isolated protamine and Sigma clupeine.

	Isolated Protamine				Sigma Clupeine			
Protein Conc. µg/mL	Trial 1		Trial 2		Trial 1		Trial 2	
0	2.238	2.254	1.813	1.869	2.250	2.252	1.725	1.761
25	2.129	2.208	1.618	1.670	2.224	2.232	1.805	1.815
50	0.070	0.168	1.473	1.500	0.074	1.525	1.692	1.571
100	0.093	0.070	0.624	0.070	0.075	0.082	0.230	0.407
200	0.075	0.075	0.046	0.061	0.081	0.081	0.106	0.062
400	0.061	0.066	0.054	0.054	0.072	0.079	0.051	0.062

Table A.11: Optical density (A625) readings obtained for *Bacillus subtilis* grown in the presence of different concentrations of isolated protamine and Sigma clupeine.

	Isolated Protamine				Sigma Clupeine			
Protein Conc. µg/mL	Trial 1		Trial 2		Trial 1		Trial 2	
0	0.883	0.881	0.870	0.880	0.846	0.858	0.886	0.910
25	0.070	0.104	0.148	0.099	0.074	0.108	0.089	0.150
50	0.070	0.073	0.069	0.048	0.110	0.110	0.052	0.052
100	0.085	0.078	0.060	0.056	0.156	0.134	0.059	0.052
200	0.079	0.080	0.051	0.052	0.166	0.197	0.059	0.059
400	0.060	0.068	0.038	0.047	0.224	0.180	0.050	0.062

Table A.12: Optical density (A625) readings obtained for *Escherichia coli* grown in the presence of different concentrations of isolated protamine or Sigma clupeine.

	Isolated Protamine				Sigma Clupeine			
Protein Conc. µg/mL	Trial 1		Trial 2		Trial 1		Trial 2	
0	1.427	1.472	1.388	1.374	1.373	1.444	1.412	1.388
25	0.103	0.223	0.070	0.050	1.274	0.134	0.701	1.347
50	0.064	0.061	0.052	0.044	0.155	0.158	0.045	0.052
100	0.072	0.065	0.053	0.050	0.198	0.187	0.052	0.056
200	0.071	0.069	0.044	0.049	0.228	0.225	0.054	0.059
400	0.061	0.064	0.040	0.043	0.223	0.219	0.042	0.038

Table A.13: Number of *Listeria monocytogenes* 4b (CFU/mL) obtained by aerobic plate count after treatment with isolated protamine or Sigma clupeine.

	Isolated Protamine				Sigma Clupeine			
Protein Conc. µg/mL	Trial 1		Trial 2		Trial 1		Trial 2	
0	4.25E8	3.30E8	3.35E8	1.80E8	4.05E8	4.70E8	3.55E8	3.35E8
25	3.40E8	3.10E8			3.60E8	4.45E8		
50	6.65E8	6.25E8			7.50E8	4.25E8		
100	6.65E8	8.45E8	9.45E8	9.65E8	7.50E8	6.05E8	8.70E8	9.50E8
200	1.04E9	1.03E9	1.39E9	1.07E9	1.18E9	1.05E9	1.09E9	1.03E9
400	2.65E6	4.85E6	2.00E6	2.65E6	1.11E8	3.10E6	2.10E8	1.70E8
800	8.72E3	1.08E4	1.02E5	4.06E3	1.19E5	3.00E5	5.50E4	1.75E4

Table A.14: Optical Density (A625) readings obtained for *Pseudomonas fluorescens* grown in the presence of different concentrations of isolated protamine and Sigma clupeine.

	Isolated Protamine				Sigma Clupeine			
Protein Conc. µg/mL	Trial 1		Trial 2		Trial 1		Trial 2	
0	1.052	1.048	1.478	1.307	1.180	1.198	1.469	1.587
25	0.898	0.910	0.926	1.028	0.877	0.898	1.355	1.229
50	0.700	0.680	1.055	1.125	0.842	0.811	1.296	1.040
100	0.187	0.167	0.228	0.266	0.460	0.405	0.409	0.453
200	0.054	0.052	0.081	0.080	0.103	0.103	0.091	0.083
400	0.046	0.045	0.063	0.055	0.110	0.110	0.061	0.065

Table A.15: Optical density (A625) readings obtained for *Salmonella typhimurium* grown in the presence of different concentrations of isolated protamine and Sigma clupeine.

	Isolated Protamine				Sigma Clupeine			
Protein Conc. µg/mL	Trial 1		Trial 2		Trial 1		Trial 2	
0	0.831	0.820	0.892	1.015	0.847	0.893	0.968	0.953
25	0.761	0.778	0.901	0.930	0.811	0.797	0.950	0.975
50	0.325	0.655	0.051	0.042	0.689	0.697	0.823	0.807
100	0.058	0.060	0.053	0.046	0.166	0.167	0.050	0.051
200	0.052	0.050	0.050	0.051	0.179	0.180	0.047	0.053
400	0.042	0.050	0.039	0.043	0.156	0.167	0.036	0.041

Table A.16: Optical Density (A625) readings obtained for *Aeromonas sobria* grown in the presence of different concentrations of isolated protamine and Sigma clupeine.

	Isolated Protamine				Sigma Clupeine			
Protein Conc. µg/mL	Trial 1		Trial 2		Trial 1		Trial 2	
0	1.110	1.120	1.615	1.646	1.135	1.130	1.641	1.660
25	1.207	1.103			1.074	1.056		
50	1.155	1.155	1.435	1.432	1.107	1.104	1.557	1.542
100	1.089	1.102	1.177	1.290	1.098	1.117	1.330	1.317
200	0.955	0.228	0.235	0.353	0.972	0.945	0.566	0.560
400	0.435	0.079	0.046	0.041	0.923	0.143	0.060	0.064
600			0.037	0.037			0.045	0.040
800			0.032	0.033			0.039	0.040
1000			0.030	0.026			0.037	

Table A.17: Optical density (A625) readings obtained for *Staphylococcus aureus* grown in the presence of different concentrations of isolated protamine and Sigma clupeine.

	Isolated Protamine				Sigma Clupeine			
Protein Conc. µg/mL	Trial 1		Trial 2		Trial 1		Trial 2	
0	1.011	1.066	0.827	0.863	0.978	1.061	0.911	0.916
25	0.071	0.062	0.059	0.054	0.134	0.120	0.047	0.052
50	0.076	0.074	0.068	0.069	0.084	0.156	0.054	0.064
100	0.080	0.078	0.080	0.079	0.205	0.199	0.070	0.072
200	0.072	0.072	0.085	0.080	0.218	0.247	0.085	0.070
400	0.052	0.063	0.063	0.075	0.216	0.223	0.072	0.066

Table A.18: Optical Density (A625) readings obtained for *Vibrio anguillarum* grown in the presence of different concentrations of isolated protamine or Sigma clupeine.

	Isolated Protamine				Sigma Clupeine			
Protein Conc. µg/mL	Trial 1		Trial 2		Trial 1		Trial 2	
0	0.340	0.362	0.460	0.582	0.355	0.353	0.415	0.449
25	0.070	0.062	0.165	0.041	0.090	0.115	0.050	0.090
50	0.063	0.063	0.043	0.040	0.072	0.079	0.040	0.036
100	0.069	0.070	0.039	0.038	0.076	0.088	0.038	0.040
200	0.070	0.066	0.039	0.035	0.073	0.077	0.036	0.036
400	0.060	0.060	0.036	0.035	0.079	0.074	0.038	0.032

Table A.19: Optical density (A625) readings obtained for *Yersinia ruckerii* grown in the presence of different concentrations of isolated protamine and Sigma clupeine.

	Isolated Protamine				Sigma Clupeine			
Protein Conc. µg/mL	Trial 1		Trial 2		Trial 1		Trial 2	
0	1.000	1.068	0.860	0.800	0.997	1.018	0.852	0.822
100	0.754	0.758	0.766	0.743	0.717	0.727	0.697	0.721
200	0.648	0.635	0.694	0.711	0.588	0.623	0.742	0.710
400	0.571	0.562	0.617	0.574	0.538	0.556	0.607	0.609
600	0.430	0.461	0.550	0.515	0.463	0.440	0.554	0.554
800	0.357	0.388	0.440	0.398	0.279	0.308	0.340	0.378
1000	0.279	0.254	0.079	0.086	0.033	0.039	0.048	0.034
1200			0.051	0.051			0.029	0.031
1400			0.045	0.043			0.032	0.032