PURIFICATION AND PARTIAL CHARACTERIZATION OF A MYOFIBRIL-BOUND SERINE PROTEASE AND ITS ENDOGENOUS INHIBITOR FROM SKELETAL MUSCLE OF THE OSTRICH

by

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DECLARATION

I declare that the dissertation hereby submitted by me, **Shonisani Cathphonia Tshidino**, for the requirements for the degree of Magister Scientiae (MSc) in the Faculty of Science at Nelson Mandela Metropolitan University, is my own work and that I have not previously submitted the same work for a degree at/ in another university/ faculty.

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ACKNOWLEDGEMENTS

DEDICATION

I dedicate this study to my father Mr. Ravhambelani Richard Tshidino and my mother Mrs. Muofhe Salminah Mukovhi, who have been my inspirations in life. I also dedicate this study to my younger sisters Hulisani Sister, Nndivhuweni Marvelous, Tshilidzi Marcia, and my brother Fhumulani Kipson Tshidino and my uncle Mpfariseni George Mukovhi and his family.

SUMMARY

The ostrich is becoming an important source of meat for humans in developed and developing countries. This study was conducted to purify and characterize myofibril-bound serine protease (MBSP) and its endogenous inhibitor (MBSPI) from skeletal muscle of the ostrich. It is well documented that MBSP is tightly bound to myofibrils and its endogenous inhibitor has been purified from the same tissue of other studied mammalian species. Literature supports an association of MBSP and its endogenous inhibitor with the degradation of myofribrillar proteins, resulting in the softening of muscle that lead to the conversion of muscle into meat with the control of the inhibitor.

MBSP was successfully dissociated from washed myofibrils by 40% ethylene glycol at pH 8.5. Following centrifugation, MBSP was partially purified in two chromatographic steps, namely Toyopearl Super Q 650S and p-aminobenzamidine-Agarose. On the other hand, MBSPI was fractionated from the sarcoplasmic fraction using 75% ammonium sulfate saturation, followed by centrifugation and partially purified by three chromatographic steps, namely Toyopearl Super Q 650S, Superdex 200 and HiTrap SP HR. Ostrich MBSP was physicochemically and kinetically characterized, while MBSPI was only physicochemically characterized.

Ostrich MBSP revealed an M_r of 21 kDa, cleaving synthetic fluorogenic substrates specifically at the carboxyl side of arginine residues. Optimum pH and temperature of ostrich MBSP were 8.0 and 40˚C, respectively. Kinetic parameters (K_m and V_{max} values) were calculated from Lineweaver-Burk plots. The characteristics of ostrich MBSP were compared to the values obtained for commercial bovine trypsin in this study, as well as that obtained for MBSP from various fish species and mouse. The results suggest that ostrich MBSP is a trypsin-like serine protease, thereby confirming the existence of MBSP in ostrich skeletal muscle. Partially purified ostrich MBSPI (M_r 17 kDa) (one form) shares 100% identity to myoglobin from the same species, while 2 other forms of MBSPI (M_r values of 35 and 36 kDa) exhibited high sequence identity to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (76%) from human and rat.

Keywords: ostrich, myofibril-bound serine protease, myofibrils, endogenous inhibitor.

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ABBREVIATIONS

A_{280nm}	Absorbance at 280nm
A_{570nm}	Absorbance at 570nm
AmAc	Ammonium acetate
APS	Ammonium persulfate
AS	Ammonium sulfate
BAPNA	Benzoyl-DL-arginine-p-nitroanilide-HCl
BCA	Bicinchioninic acid
Boc	*t*-butyloxycarbonyl
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue R-250
CNBr	Cyanogen bromide
DFP	Diisopropyl fluorophosphate
dH_2O	distilled water
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
Emλ	Emission wavelength
Exλ	Excitation wavelength
FPLC	Fast protein liquid chromatography
h	hour(s)
HA	Hydroxylapatite

HMW	High molecular weight
IEC	Ion-exchange chromatography
kDa	Kilodalton
K_m	Michaelis-Menten constant
LMW	Low molecular weight
MBSP	Myofibril-bound serine protease
MBSPI	Myofibril-bound serine protease inhibitor
MCA	4-methylcoumaryl-7-amide
min	minute(s)
ml	millilitter
M_r	Relative molecular weight(s)
$Na_2B_4O_7\text{-}KH_2PO_4$	Sodium tetra borate-potassium dihydrogen phosphate
PEG	polyethylene glycol
PMSF	Phenyl methyl sulfonyl fluoride
PVDF	Polyvinylidene difluoride
R_m	Relative mobility
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
s	seconds
Suc	Succinyl
TEMED	N, N, N, N-tetramethylethylenediamine
Tris	2-amino-2-(hydroxymethyl)-1, 3-propanediol

V_{max}	maximum velocity
V	Voltage
λ	wavelength

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CHAPTER 1: LITERATURE SURVEY

1.1. INTRODUCTION

Meat largely reflects the chemical and structural nature of the muscle. Meat differs from muscle by a series of biochemical and biophysical changes, which are initiated in muscles at the death of the animal (Lawrie, 1991). The conversion of muscle into meat is a complex process in which all mechanisms responsible for the development of meat qualities are very likely interdependent. Proteolysis is probably the process involved in the development of meat tenderness (Ouali *et al.,* 2006). Conversion of muscle into meat is a three-step process. The pre-rigor phase, during which muscle remains excitable, might correspond to the duration of survival of the nervous system. It has been found that most changes in post-rigor meat texture result primarily from a weakening of the myofibrillar structure (Sentandreu *et al.,* 2002). The onset and extent of rigor mortis are biochemically characterized by the content of energy-rich compounds, including ATP, creatine phosphate and glycogen, together with ATPase, kinase and glycolytic enzyme activities in the muscle (Lawrie, 1991). Upon rigor onset, muscle elasticity decreases and at its completion, the tissue reaches its maximum toughness. The last step is the tenderizing phase that differs in length according to the chilling conditions as well as between muscles, individual animals and animal species.

Proteolysis of muscle proteins appears to be the major contributor to the tenderization process during post-mortem ageing (Van Jaarsveld *et al*., 1997). In meat, the events that occur in the skeletal muscle during the maturation and conditioning period should be identical to those occurring in the cardio-vascular system during the ischemic period. Nevertheless, today almost nothing is known about the mechanisms which regulate oxidative deterioration and particularly the oxidation of muscle proteins during meat maturation. It has been observed that physiological processes such as oxidative processes determined in sarcoplasmic and myofibrillar proteins could be implicated in colour and tenderness changes during maturation, respectively (Renerre *et al.*, 1996). The maturation of meat is known as the transformation of the muscle into meat, which is associated with a decrease in the pH from 7.0-7.2 to 5.4-5.8 (Lawrie 1991; Ortiques-Marty *et al*., 2006).

The optimum pH for stability of myofibril-bound serine protease (MBSP) is mostly known to be around 6.0-9.0 (Ohkubo *et al*., 2004a, 2004b; Ohkubo *et al.*, 2005; Cao *et al*., 2006a; Osatomi *et al*., 1997). Jiang (1998) discussed the different biochemical and ultra structural changes occurring in the maturation process, as well as myofibrillar disruption and changes in contractile proteins. The skeletal muscle contains multiple mechanisms for the degradation of myofibrillar proteins *in vivo* and both lysosomal and nonlysosomal pathways are responsible (Guo *et al*., 2007). The lysosomal pathways include cysteine proteases, calpains, proteasomes and other enzymes such as sarcoplasmic serine proteases (SSP) and MBSPs. Except calpains, proteases involved in the nonlysosomal pathway have been characterized as serine proteases. In the skeletal muscle of various fish species, rat and mouse, the existence of serine proteases in the sarcoplasmic fraction and myofibrillar fraction has been identified (Cao *et al*., 2005).

1.2 Proteases

Protease is synonymous with peptide hydrolase and this term includes all enzymes that cleave peptide bonds. Proteases are subdivided into exopeptidases, whose action is directed to the amino or carboxyl terminus of the protein or peptide, and endopeptidases, enzymes that cleave peptide bonds internally in peptides and usually cannot include the amino or carboxyl terminal amino acids at the active site (Bond and Butler, 1987; Elliot and Elliot, 2001). Barrett and McDonald (1986) suggested that the term endopeptidase be used synonymously with proteinase. All proteases have been found to exist intracellularly at some stage. Normally mammalian cellular proteases are integral components of cells. However, these intracellular proteases may act on intracellular or extracellular peptides or proteins in association with a cell structure or compartment (Bond and Butler, 1987). Proteases are able to activate or inactivate biologically active proteins and peptides by limited proteolysis and favour to regulate diverse biological processes (Fisher *et al.,* 1983).

1.2.1 General structural features of proteases and their inhibitors

Most of the proteases are molecules of relatively small size and of compact, nearly spherical structure. Proteases are generally the simple enzymes, which are devoid of allosteric regulatory mechanisms. The latter property is usually connected with structures made up of a number of subunits. None of the proteases possesses such a structure, all being made up of a single unit. However, in some of them there is more than one polypeptide chain; nevertheless, these are produced from a single chain of the zymogen during the activation process (Mihalyi, 1972). Most protease inhibitors have one or two disulfide bridges (Bernard and Peanasky, 1993). Protein protease inhibitors are utilized as affinity supports for isolation of serine proteases (Laskowski *et al*., 1989).

1.2.2 Classification of proteases and their inhibitors

Proteases are further classified into four prominent groups based on the functional group present at their active sites; these are serine proteases, aspartic proteases, cysteine proteases and metalloproteases (Rawlings and Barrett, 1993). All protease groups execute a large variety of complex physiological functions. Their importance in conducting such an essential metabolic and regulatory role is evident from their occurrence in all forms of living organisms. Proteases play a critical role in many physiological processes including protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis, inactivation of zymogens, and transport of secretory proteins across membranes (Rawlings and Barrett, 1993). Cysteine proteases were formerly called thiol proteases containing an essential cysteine residue that is involved in a covalent intermediate thioester complex with substrates. Cysteine proteases are known to be inhibited by low concentrations of *p*-hydroxymercuribenzoate (*p*HMB), which is the hydrolysis product of *p*-chloromercuribenzoate, and alkylating reagents such as iodoacetate, iodoacetamide and *N*-ethyl maleimide (NEM). Aspartic proteases were formally known as acid proteases. They contain two aspartic acid residues at their active sites that are involved in catalysis and are known to be inhibited specifically by pepstatins and diazoacetyl compounds, such as diazoacetyl-L-Phe-methyl ester. Metalloproteases contain metal ions, usually zinc at

the active site. The metal ions are an integral part of their structure, which enhance the nucleophilicity of water and polarize the peptide bond prior to be cleaved by nucleophilic attack. Metalloproteases are inhibited by chelating agents such as ethylenediamine tetraacetic acid (EDTA) and 1, 10-phenanthroline (Bond and Butler, 1987). The study of the inhibition of enzymes is one of the most extensively studied fields of enzymology. The importance of an inhibition reaction is of a more practical nature and is connected to the need of stopping the enzymatic reaction at certain intermediary stages, for the purpose of studying the kinetics of the reaction, the appearance of intermediates and other aspects. For this application, the inhibition must be rapid, complete and preferably irreversible (Mihalyi, 1972).

Protease inhibitors are widely distributed in plants, microorganisms and animal tissues. In the past few years the interest in understanding their physiological roles has increased, because of their important function in the regulation of various processes in which proteases are involved, such as intracellular protein breakdown, transcription, cell cycle, cell invasion and many other processes (Sangorrin *et al.,* 2001). With the exception of macroglobulins, which inhibit proteases of all classes, individual inhibitors only inhibit proteases belonging to a single mechanistic class; serine protease inhibitors can be utilized as drugs. Their use is frequently limited by the relatively low specificity of inhibitors revealing the isolation and inhibition of unwanted enzymes along with the intended ones (Laskowski and Iknoshin, 1980).

The inhibition of proteases can be classified in several categories according to their mode of action (Mihalyi, 1972):

- Inhibitors irreversibly destroying the enzyme; these include all the physical and chemical agents that are capable of denaturing the enzyme, e.g. heat and protein precipitation by ethanol, trichloroacetic acid, perchloric acid, acetone, etc.
- Noncompetitive reversible inhibitors in which a series of substances can interact nonspecifically with the enzyme and lead to a decrease in its catalytic activity.

- Competitive reversible inhibitors; these include substances that are capable of binding to the active center of an enzyme without undergoing catalytic decomposition, thereby preventing binding of a substrate.
- Active-site directed, irreversible inhibitors; these reagents react irreversibly with one of the residues forming the active site of the enzyme.
- Inhibition of specific enzymes.

1.2.3 Serine proteases

Serine proteases are a family of enzymes that catalyze the cleavage of specific peptide bonds in proteins and peptides (Copeland, 2000). These enzymes are characterized by the presence of a uniquely reactive serine side chain at the active centre that reacts irreversibly with organophosphates such as diisopropyl fluorophosphate, and the catalytic mechanism of these proteases involves the covalent binding of substrate to a serine residue (Bond and Butler, 1987).

1.2.3.1 Regulation of serine proteases

Physiological regulation is defined as a matter of activation and inhibition of enzymes. Many physiological reactions are regulated by the proteolytic conversion of an inactive precursor of a protein, which is a zymogen, to an active form. Protease inhibitors of the serpin super family play important roles in regulating the serine proteases of blood coagulation, fibrinolysis, inflammation and many other physiological processes (Olson *et al.,* 1995). These serine protease inhibitors are single chain proteins of approximately 400 amino acid residues and are therefore larger than other non-serpin inhibitors, containing from 29 to 190 amino acid residues. Therefore, serpin and non-serpin inhibitors inhibit proteases by forming stable equimolar complexes in which a substrate-like interaction is made between an exposed inhibitor-binding loop and the enzyme active site (Olson *et al.,* 1995).

1.2.3.2 Pancreatic serine proteases

The mammalian serine proteases were found to have a common tertiary structure as well as a common function. There are three major pancreatic enzymes, namely trypsin, chymotrypsin and elastase. These enzymes are kinetically similar, as they all catalyze the hydrolysis of peptide and synthetic ester substrates. Their activity peak has been resolved at around pH 7.8 and at a lower pH with a pK_a of approximately 6.8. In these conditions, an "acylenzyme" is formed via esterification of the hydroxyl of the reactive serine by the carbonyl portion of the substrate. The major difference among these three enzymes is specificity.

Trypsin is specific for peptides and esters of the amino acids lysine and arginine, while chymotrypsin is specific for large hydrophobic side chains of phenylalanine, tyrosine and tryptophan. On the other hand, elastase is specific for small hydrophobics, such as alanine. The polypeptide backbones of all three enzymes are essentially superimposable. These specificity differences lie mainly in a pocket that binds the amino acid side chains as shown in Figure 1.1 (Elliott and Elliott, 2001; Fersht, 1999).

1.2.3.3 Mechanism of action of serine proteases

Serine proteases hydrolyze peptides and synthetic ester substrates by the acylenzyme mechanism. This mechanism is initiated by the association of the enzyme and substrate forming a nonconvalent enzyme-substrate complex, held together by physical forces of attraction, followed by the attack of the hydroxyl of Ser-195 on the substrate giving the first tetrahedral intermediate. The intermediate collides to give the acylenzyme releasing the amine or alcohol. The acylenzyme hydrolyses to form the enzyme-product complex through a second tetrahedral intermediate (Figure 1.2) (Fersht, 1999). The residues that function in the active site include His-57, Asp-102 and Ser-195. This mechanism depends on the fact that His-57 lies sufficiently close to Ser-195 for one of its nitrogens to form a hydrogen bond with the hydroxyl of Ser-195. Asp-102 is able to form another hydrogen bond with the other ring nitrogen of His-57. A negative charge is displaced from Asp-102 towards the hydroxyl of Ser-195, making this hydroxyl highly reactive (Campbell and

Smith, 2000). Comparison of the binding pockets in chymotrypsin, elastase, and trypsin is shown in Figure 1.1
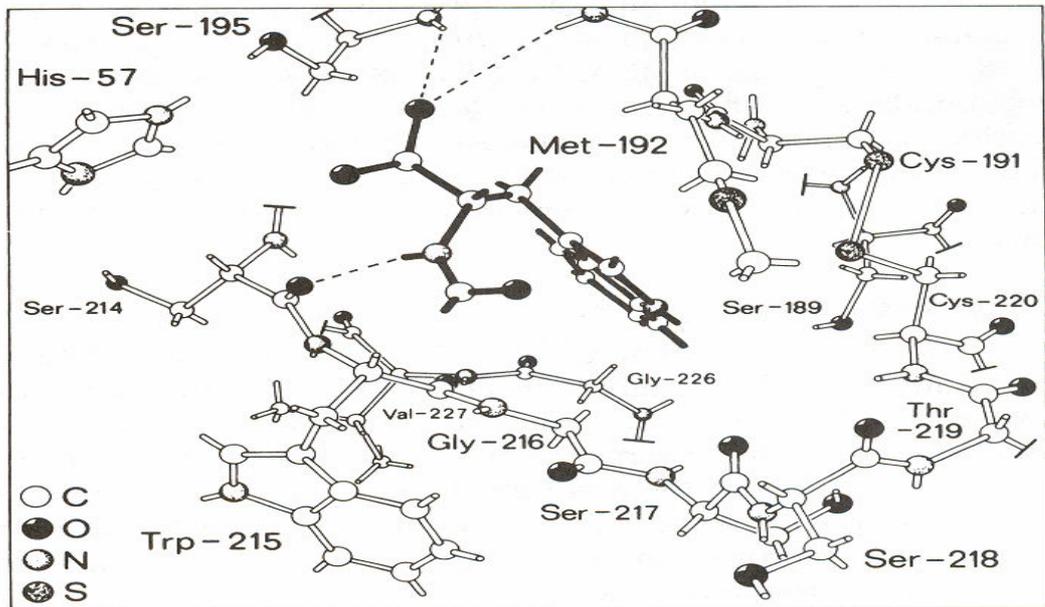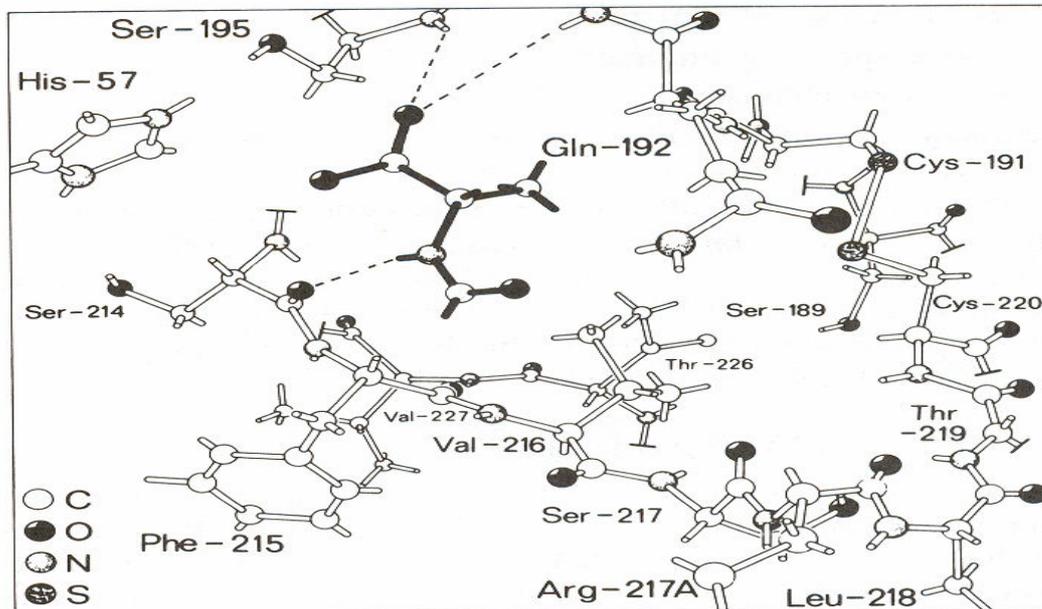
Figure 1.1. Comparison of the binding pockets in (a) chymotrypsin, with N-formyl-L-tryptophan bound, and (b) elastase, with N-formyl-L-alanine bound. The binding pocket in trypsin is very similar to that in chymotrysin, except that residue 189 is an aspartate in trypsin, binding positively charged side chains. Note the hydrogen bonds between the substrate and backbone of the enzyme (Taken from Fersht, 1999).

The reaction mechanism of chymotrypsin is shown in Figure 1.2.
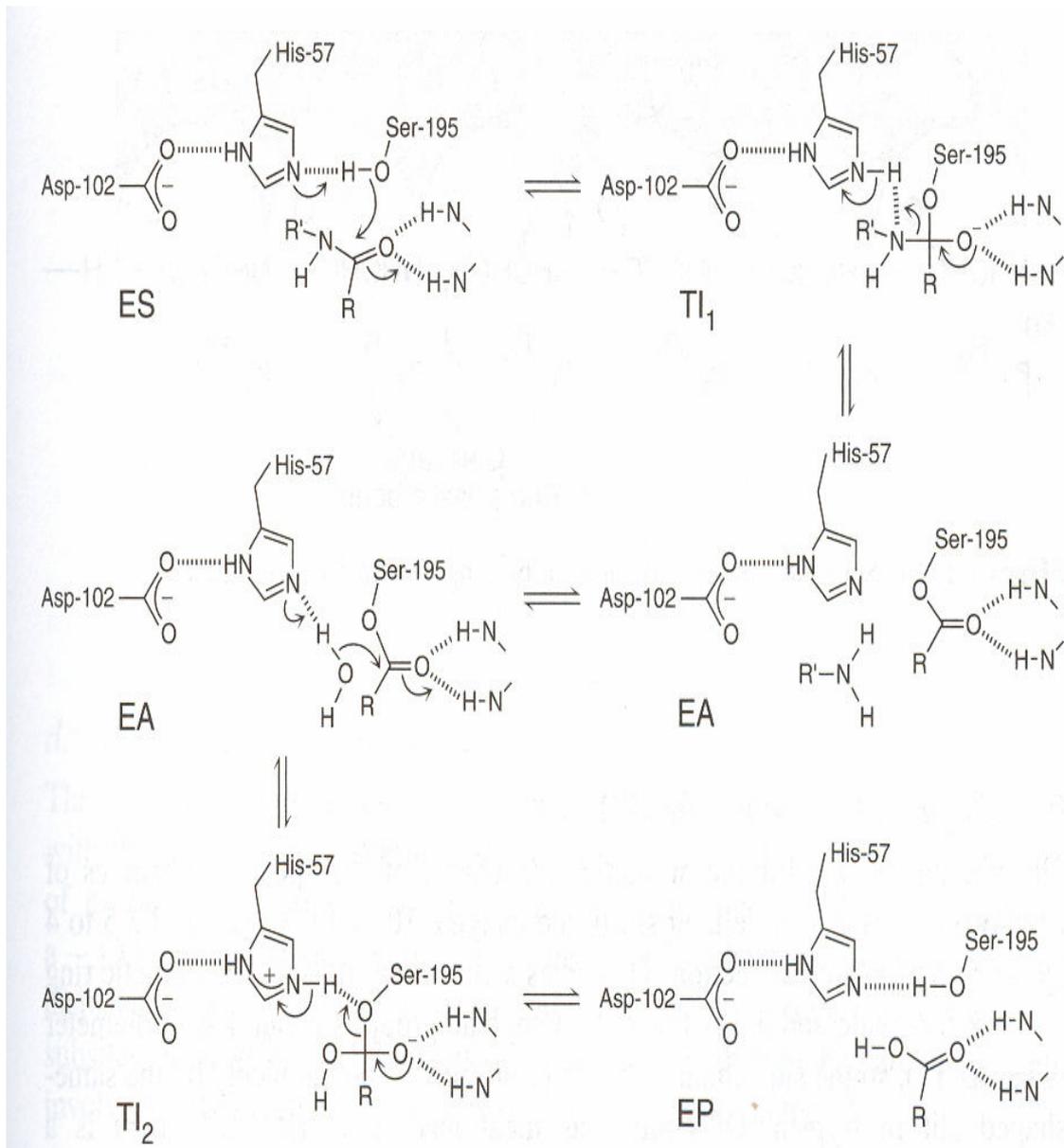
Figure 1.2. Reaction mechanism of chymotrypsin (Taken from Fersht, 1999)

1.3 MBSP and its endogenous inhibitor (MBSPI) in skeletal muscles

Earlier studies on MBSP and MBSPI were focused on food chemistry, because they have been a candidate enzyme and inhibitor inducing a "modori" (Cao *et al*., 1999a; Jiang *et al.,* 2006; Osatomi *et al.*, 1997). The modori phenomenon (thermal gel degradation of fish jelly production) had led to research focusing on isolation of the enzymes involved in the manufacturing process of fish jelly products. Serine proteases have been purified from skeletal muscle of many fish species and all of them were isolated and purified from the sacroplasmic fraction (Cao *et al.,* 2000a; Ohkubo *et al.,* 2004a, 2004b; Osatomi *et al.*, 1997; Yanagihara *et al.*, 1990). Serine protease inhibitors have greatly suppressed the degradation of MHC by MBSP, while other inhibitors of cysteine, metallo and aspartic proteases showed only partial or complete inhibitory effects (Cao *et al*., 2006b). The effects of soybean trypsin inhibitor (STI) on the degradation of crucian carp myofibrillar proteins caused by an endogenous serine protease have been studied (Jiang *et al.,* 2006).

MBSP in white croaker skeletal muscle has been detected (Ohkubo *et al*., 2005) and its endogenous inhibitor purified, and submitted for protein sequence analysis (Cao *et al*., 2000c). Sangorrin *et al.* (2000, 2001, 2002) described the variability of a serine protease (protease M) in mouse muscle with a high molecular weight (M_r) value of 120 kDa, showing a great capacity to degrade whole myofibrils *in vitro,* and its endogenous inhibitor. The endogenous inhibitor has been characterized as a glycoprotein with a high M_r value of 110 kDa, which prevented the action of the protease, both on natural and artificial substrates (Sangorrin *et al.*, 2002). The enzyme characteristics and the sequence alignment combinations revealed that carp MBSP is an unique trypsin-like serine protease, which is expressed in normal skeletal muscle (Guo *et al.,* 2007). A novel MBSPI from lizardfish skeletal muscle was purified to homogeneity and has been regarded as a specific endogenous inhibitor of MBSP (Cao *et al.*, 2000b, 2001). A number of purification procedures for MBSP and MBSPI, involving ion exchange, hydrophobic interaction, affinity and gel exclusion chromatographies have been well documented. The isolation, purification procedures and characteristics of MBSPs from ordinary and skeletal muscles of various fish species and mouse are summarized in Table 1.1.

Table 1.1: Purification procedures and characteristics of MBSP from ordinary and skeletal muscles of various fish species and mouse.

Species	Skeletal muscle (kg)	MBSP Extraction method	Chromatographic steps	Yield (µg)	MBSP M_r (kDa)	Specific enzyme activity (U/mg)
White croaker (***Argyrosmus argentatus***)[a]	2	Heat treatment	Sephacryl S-300; Benzamidine-Sepharose 6B	1.1	67 (monomer)	10909
Yellow croaker (***Pseudosciaena crocea***)[b]	0.3	Heat treatment	Sephacryl S-200; Mono Q (FPLC); Bio-Sil SEC-125 (HPLC)	300	94 and 34 (homodimer)	-
Lizard fish (***Saurida wanieso***)[c]	0.5	Heat treatment	DEAE-Sephacel; Sephacryl S-200; Q-Sepharose; Hydroxylapatite	<50	60 and 29 (homodimer)	72
Lizard fish *(Saurida undosquamis)*[d]	2	Heat treatment	Q-Sepharose; Phenyl-Sepharose	370	50 and 28 (homodimer)	206.1
Mouse[e]	14 x 0.25 g (body weight)	40% ethylene glycol	Mono Q HR (FPLC); Superose 12 (FPLC)	240	120 (monomer)	150
Carp (***Cyprinus carpio***)[f]	3	KCl	Ultrogel AcA 54; Sephadex G-25; Arginine-Sepharose 4B	74.8	30 (monomer)	3260
Silver carp[g]	0.3	Acid treatment	Sephacryl S-200; High Q; Arginine Sepharose-4B	100	28 (monomer)	580
Crucial carp (***Carassius auratus***)[h]	0.3	Acid treatment	Q-Sepharose; Benzamidine-Sepharose 6B; Sephacryl S-200	1200	28 (monomer)	15.6

a, Ohkubo *et al.* (2005); b, Cao *et al.* (2006a); c, Cao *et al.* (2000a); d, Ohkubo *et al.* (2004a); e, Sangorrin *et al.* (2000, 2002); f, Osatomi *et al.* (1997); g, Cao *et al.* (2005); h, Guo *et al.* (2007). All purified MBSPs were of trypsin-type serine proteases. These enzymes were also characterized by an optimum pH and temperature of 8 and 55°C, respectively.

The isolation, purification procedures and characteristics of MBSPs and its endogenous inhibitor from ordinary and skeletal muscles of various fish species and mouse are summarized in Table 1.2.

Table 1.2: Purification of MBSP and its endogenous inhibitor from muscles of various fish species and mouse.

Species	Skeletal muscle (kg)	MBSP M_r (K)	MBSPI extraction	Chromatographic steps	Yield (µg)	MBSP M_r (kDa)
White croaker (*Argyrosomus argentatus*) [ab]	2 for MBSP 0.5 for MBSPI	67 (monomer)	$(NH_4)_2SO_4$ precipitation (45-75% saturation)	DEAE-Sephacel; Heat treatment (45˚C, 1 min); SP-Sepharose; Sephadex G-150; HPLC	-	55
White croaker (*Micropogon opercularis*) [c]	0.3 for MBSPI		2% KCl	DEAE-Sephacel; Con A Superose; Sephacryl S-300; Mono Q (FPLC)	600	65
Lizard fish (*Saurida wanieso*) [de]	0.5 for MBSP O.5 for MBSPI	60 29 (homodi-mer)	$(NH_4)_2SO_4$ precipitation (75% saturation)	DEAE-Sephacel; Heat treatment (50˚C, 1 min); SP-Sepharose; Sephadex G-150	3300	50
Mouse [f]	14 x 0.25 (body weight)	120	2% KCl	Mono Q; Superose 12; Con A Superose	390	110

a, Ohkubo *et al.* (2005); b, Cao *et al.* (2000b); c, Sangorrin *et al.* (2001); d, Cao *et al.* (2000a); e, Cao *et al.* (200l); f, Sangorrin *et al.* (2000, 2002).

A summary of specific cleavage sites of peptides by purified carp MBSP is shown in Table 1.3.

Table 1.3. Summary of cleavage sites of peptides by purified carp MBSP (Taken from Cao *et al.,* 2000a).

Neurotensin

Pyr-Leu-Tyr-Glu-Asn-Lys-Pro-**Arg**▼**Arg**-Pro-Tyr-Ile-Leu

BAM-12P

Tyr-Gly-Gly-Phe-Met-**Arg**▼**Arg**-Val-Gly-**Arg**-Pro-Glu

Dynorphin A 1-13

Tyr-Gly-Gly-Phe-Leu-**Arg**▼**Arg**▼Ile-**Arg**-Pro-Lys-Leu-Lys

Vasoactive Intestinal Peptide (VIP)

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-**Arg**-Leu-**Arg**▼Lys-Gln-Met-Ala-Val-**Lys**-**Lys**-Tyr-Leu-Asn-Ser-Ile-Leu-Asn(NH$_2$)

α-Neoendorphin

Tyr-Gly-Gly-Phe-Leu-**Arg**▼**Lys**-Tyr-Pro-Lys

Peptides not cleaved

Lysyl-Bradykinin

Lys-**Arg**-Pro-Pro-Gly-Phe-Ser-Pro-Phe-**Arg**

Methionyl-Lysyl-Bradykinin

Met-Lys-**Arg**-Pro-Pro-Gly-Phe-Ser-Pro-Phe-**Arg**

The sites and extent of peptide cleavage by purified lizard fish (*S. wanieso*) MBSP in each peptide are shown. A large arrowhead indicates a major cleavage site, and a small arrowhead, a minor cleavage site.

The hydrolysis of peptides showed that the purified lizard fish MBSP selectively cleaved at the arginine residue of the bonds between two consecutive residues (Arg-Arg and Arg-Lys), whereas Lys-Arg pair in lysyl-bradykinin, methionyl-lysyl-bradykinin and Lys-Lys pair in vasoactive intestinal peptide (VIP) were not at all affected which further exhibited its arginine-specific cleavage characteristic. This result corresponded well with those

Alignment of the N-terminal amino acid sequence of MBSP with other enzymes is shown in Figure 1.3.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
MBP	Lizard fish MBSP[a]	I	V	G	G	Y	E	X	E	A	Y	S	K	P	Y	Q	V	S	I	N	L	G	Y
	Carp MBSP[b]	I	I	G	G	Y	E	X	K	P	H	S	Q	P	W	Q	A	F	L	V	D	N	K
	Lizard fish MBSP[c]	I	V	G	G	A	E	X	V	P	Y												
Trypsin	Carp A[d]	I	V	G	G	Y	E	X	E	P	Y	S	T	P	W	Q	A	S	L	N	S	G	Y
	Carp B[d]	I	V	G	G	Y	E	X	T	P	H	S	Q	P	W	Q	V	S	L	N	S	G	Y
	Salmon[e]	I	V	G	G	Y	E	C	K	A	Y	S	Q	P	H	Q	V	S	L	N	S	G	Y
	Cod[f]	I	V	G	G	Y	E	C	T	R	H	S	Q	A	H	Q	V	S	L	N	S	G	Y

Figure 1.3. Comparison of the N-terminal amino acid sequence of purified MBSP from lizard fish with other MBSPs and various fish trypsins. a. *S. undosquamis* MBSP; b. carp MBSP; c. *S. wanieso* MBSP; d. carp trypsin A, B; e. salmon trypsin; f. cod trypsin (Taken from Ohkubo *et al*., 2004a).

The N-terminal amino acid sequence of lizard fish MBSP (22 amino acid residues) was compared with those of fish trypsins and other MBSPs. The sequence of purified lizard fish (*S. undosquamis*) MBSP showed a high homology to fish trypsins, and lizard fish (*S. wanieso*) MBSP, while it exhibited low homology to carp MBSP

Alignment of primary structures of mature serine proteases with those from other sources is shown in Figure 1.4.

```
crucian carp MBSP      IIGGYECRPHSQPWQAFLTDN.....RISCGGSLINERWA    35
chum salmon trypsin    IVGGYECKAYSQPHQVSLNSG.....YHFCGGSLVNENWV    35
cod trypsin-I          IVGGYECTKHSQAHQVSLNSG.....YHFCGGSLVSKDWV    35
porcine trypsin        IVGGYTCAANSIPYQVSLNSG.....SHFCGGSLINSQWV    35
rat trypsin-IA         IVGGYTCPEHSVPYQVSLNSG.....YHFCGGSLINDQWV    35
hamster mekratin       IIGGVESKPHSRPYMAHLEIVTERGFTASCGGFLITPEFV    40
human KLK14            IIGGHTCTRSSQPWQAALLAGPRR..RFLCGGALLSGQWV    38
                       *  **       *        *           *** *

crucian carp MBSP      VSAAHCNFQQDRLSVRLGRHNLVTAENTEQRIEAEKMIPF    75
chum salmon trypsin    VSAAHC..YKSRVEVRLGEHNIKVTEGSEQFISSSRVIRH    73
cod trypsin-I          VSAAHC..YKSVLRVRLGEHHIRVNEGTEQYISSSSVIRH    73
porcine trypsin        VSAAHC..YKSRIQVRLGEHNIDVLEGNEQFINAAKIITH    73
rat trypsin-IA         VSAAHC..YKSRIQVRLGEHNINVLEGDEQFINAAKIIKH    73
hamster mekratin       MTAAHCK..GKEITVTLGAHDVSKAESTQQKIKVKKQIAH    78
human KLK14            ITAAHCG..RPILQVALGKHNLRRWEATQQVLRVVRQVTH    76
                          ****       *  ** *        *     *

crucian carp MBSP      PKYNDRPHNNDIMLIKLKQPATLNRYVKPIPLP..NKCPS   113
chum salmon trypsin    PNYSSYNIDNDIMLIKLSKPATLNTYVQPVALP..SSCAP   111
cod trypsin-I          PNYSSYNINNDIMLIKLTKPATLNQYVHAVALP..TECAA   111
porcine trypsin        PNFNGNTLDNDIMLIKLSSPATLNSRVATVSLP..RSCAA   111
rat trypsin-IA         PNYSSWTLNNDIMLIKLSSPVKLNARVAPVALP..SACAP   111
hamster mekratin       PNYSFYSKLHDIMFLKLQKKAKEDPSVDTIPLPSPSDFLK   118
human KLK14            PNYNSRTHDNDLMLLQLQQPARIGRAVRPIEVT..QACAS   114
                       *            * *    *             *

crucian carp MBSP      AGEKCLVSGWGRTADG...IASTLQCLKLPVLSEKVCKTA   150
chum salmon trypsin    AGTMCTVSGWGNTMSS.TADKNKLQCLNIPILSYSDCNNS   150
cod trypsin-I          DATMCTVSGWGNTMSS.VADGDKLQCLSLPILSHADCANS   150
porcine trypsin        AGTECLISGWGNTKSSGSSYPSLLQCLKAPVLSDSSCKSS   151
rat trypsin-IA         AGTQCLISGWGNTLSNGVNNPDLLQCVDAPVLSQADCEAA   151
hamster mekratin       PGKMCRAAGWGRTGVT.EPTSERLREVKLRIMDKGACKN.   156
human KLK14            PGTSCRVSGWGTISSPIARYPASLQCVNINISPDEVCQKA   154
                           *    ***              *             *

crucian carp MBSP      YGSIITRNMFCAGFIRGGKDSCQGDSGGPVVCKGQLKGVV   190
chum salmon trypsin    YPGMITNAMFCAGYLEGGKDSCQGDSGGPVVCNGELQGVV   190
cod trypsin-I          YPGMITQSMFCAGYLEGGKDSCQGDSGGPVVCNGVLQGVV   190
porcine trypsin        YPGQITGNMICVGFLEGGKDSCQGDSGGPVVCNGQLQGIV   191
rat trypsin-IA         YPGEITSSMICVGFLEGGKDSCQGDSGGPVVCNGQLQGIV   191
hamster mekratin       YYHYHYDFQVCVGSPRRVRSAYKGDSGGPLLCAGVAHGIV   196
human KLK14            YPRTITPGMVCAGVPQGGKDSCQGDSGGPLVCRGQLQGLV   194
                       *          * *        ******  * *    * *

crucian carp MBSP      SFGNG.CAKPKYPGVYAEVCRYTRWIKSTIASN          222
chum salmon trypsin    SWGYG.CAEPGNPGVYAKVCIFNDWLTSTMATY          222
cod trypsin-I          SWGYG.CAERDHPGVYAKVCVLSGWVRDTMANY          222
porcine trypsin        SWGYG.CAQKNKPGVYTKVCNYVNWIQQTIAAN          223
rat trypsin-IA         SWGYG.CALPDNPGVYTKVCNFVGWIQDTIAAN          223
hamster mekratin       SYGRG...DAKPPAVFTRISSYVPWINIVIKASS         226
human KLK14            SWGMERCALPGYPGVYTNLCKYRSWIEETMRDK          227
                       * *         * *           *
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Figure 1.4. Alignment of primary structures of mature serine proteases. The mature sequence of crucial carp MBSP was compared with those from other sources (Taken from Guo *et al.*, 2007). * Identical amino acid residues.

Crucian carp MBSP shared relatively high homology to other serine proteases especially in the conserved regions (Figure 1.4). It showed 54.5% identity to chum salmon trypsin, 52.2% to cod trypsin-I, 55.1% to porcine trypsin, 54.2% to rat trypsin, 36.2% to hamster mekaratin, a chymotrypsin-type serine protease from skeletal muscle of hamster, and 47.2% to KLK14, a trypsin-type serine protease from human. The catalytic triad (His^{60}, Asp^{106} and Ser^{196}) of serine proteases is well conserved in crucian carp MBSP (Guo et al., 2007).

Alignment of tryptic peptide fragments of MBSPI with porcine phosphoglucose isomerase is shown in Figure 1.5.

```
MBSPI                LQDWY                        DNTHH
PGI   AALTQNPQFKKLQTWYHEHRSDLNLRRLFEGDKDRFNHFSLNLNTNHGRI

PGI   LLDYSKNLVTEAVMQMLVDLAKSRGVEAARERMFNGEKINFTEDRAVLHV

MBSPI                                            GYLGK
PGI   ALRNRSNTPILVDGKDVMPEVNRVLEKMKSFCKRVRSGEWKGYSGKSITD

PGI   VINIGIGGSDLGPLMVTEALKPYSAEGPRVWFVSNIDGTHIAKTLATLNP

MBSPI                          DTSEVH HFVALSTNA
PGI   ESSLFIIASKTFTTQETITNAETAKEWFLQSAKDPSAVAKHFVALSTNTT

PGI   KVKEFGIDPQNMFEFWDWVGGRYSLWSAIGLSIALHVGFDNFEQLLSGAH

MBSPI                                             XTAY
PGI   WMDQHFRTTPLEKNAPVLLALLGIWYINFFGCETHAMLPYDQYLHRFAAY

MBSPI FQQGDMES            VNYHTGPIVLGEPG                M
PGI   FQQGDMESNGKYITKSGTRVDHQTGPIVWGEPGTNGQHAFYQLIHQGTKM

MBSPI VPSD
PGI   IPCDFLIPVQTQHPIRKGLHHKILLANFLAQTEALMKGKSTEEARKELQA

MBSPI            VFQGNRPTNSIIF-K
PGI   AGKSPEDFEKLLPHKVFEGNRPTNSIVFTKLTPFILGALIAMYEHKIFVQ

MBSPI                 QLAKK
PGI   GVIWDINSFDQWGVELGKQLAKKIEPELDGSSPVTSHDSSTNGLINFIKQ

PGI   EREARSQ
```

Figure 1.5. Alignment of tryptic peptide fragments of MBSPI with porcine phosphoglucose isomerase (PGI). MBSPI was digested with carp trypsin B and sequenced (Taken from Cao *et al.*, 2000b).

Purified lizard fish MBSPI was found to be resistant to Edman degradation, suggesting its amino terminal group was blocked. Internal peptide sequences were determined using carp trypsin B digested peptide fragments separated by a reverse-phase C18 column.

Amino terminal sequences were obtained from 10 tryptic peptides. These sequences did not show homology with known serine proteinase inhibitors but revealed high identity (76%) to PGI and to PGIs from different sources such as human and mouse (Figure 1.5). Purified MBSPI revealed that PGI is a multifunctional protein which carries out isomerase activity in dimeric form, whereas its monomeric form is responsible for the neurotrophic activity. However, PGI functioning as a serine proteinase inhibitor has never been reported. MBSPI may show PGI activity indicating PGI not only catalyses the reversible isomerization of glucose 6-phosphate to fructose 6-phosphate and is responsible for neurotrophic activity, but also controls the activity of MBSP during myofibrillar protein turnover. The physiological relationship between MBSP, MBSPI and PGI is of much interest in further research (Cao *et al*., 2000b).

The biological significance of the high homology between MBSPI and PGI suggests that they are related proteins. Therefore, MBSPI possessing PGI activity is of interest. Cao *et al.* (2000b) surprisingly noticed that MBSPI showed PGI activity as the absorbance of the PGI reaction mixture increased at 340 nm in the presence of MBSPI. In order to establish if PGI exhibits inhibitory activity towards MBSP, rabbit muscle PGI was used for an investigation. Interestingly, similar to MBSPI, rabbit muscle PGI also revealed specific inhibitory activity toward MBSP. Western blotting results revealed that the relative tissue distribution of MBSPI was mainly in skeletal muscle, followed by cardiac muscle, liver and kidney. A low but detectable amount of MBSPI was observed in brain. The distribution of MBSPI was found to be very similar to that of neuroleukin, which was later proposed and further confirmed as PGI (Cao *et al*., 2000b).

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CHAPTER 2: INTRODUCTION TO THE PRESENT STUDY

The ostrich (*Struthio camelus*) is the largest of all birds and belongs to the small order of birds known as Ratitae or running birds. Ostrich farming is a rapidly growing industry in South Africa, Zimbabwe, Israel, Australia, United States, and various European countries. Ostriches are becoming an important source of meat for humans (Sales and Hayes, 1996). According to Cooper (1999), ostrich meat is perceived and marketed as a healthy alternative to other red meats. Relative to beef, ostrich meat is characterized by a higher ultimate pH (>6.2), lower collagen and higher pigment content, similar cooking loss, darker visual appearance, similar sensory tenderness, higher polyunsaturated fatty acid content and similar cholesterol content. Ostrich meat can be classified into four main categories, namely Extra class, First class, Second class (or steak) and Third class (or cook). Ostrich meat from Extra, First and Second class categories are usually commercialized as fresh meat (vacuum-packed and refrigerated or frozen) and served cooked, grilled or dried (biltong) in restaurants. Ostrich meat is highly perishable due to its high pH and the need for more research on the use of ostrich meat in value-added products is evident (Fernández-López *et al.,* 2006).

Several studies have been published on physiological properties, chemical composition, sensory properties as well as nutritive values of ostrich meat (Girolami *et al*., 2003; Sales and Hayes, 1996; Sales and Mellett, 1996; Seydim *et al*., 2006), characterized by an extremely low intramuscular fat content. Due to the emphasis placed on the nutritive value of food by consumers a great need exists for information on the nutritional composition of ostrich meat and some information on the amino acid and mineral composition of some muscles were provided (Sales *et al.,* 1996; Sales and Hayes, 1996). Such a focus is important for the success of the ostrich industry in Southern Africa (Cooper, 1999).

The present study is the first of its kind for the isolation, purification and characterization of MBSP and its endogenous inhibitor from ostrich skeletal muscle. Several studies have been conducted on muscles from various fish species in Japan and from mouse skeletal muscle in Argentina. MBSPs and their endogenous inhibitors have been purified to

homogeneity from those species. Literature supports an association of MBSP and its endogenous inhibitor with the degradation of myofribrillar proteins that results in the softening of muscle that lead to the conversion of muscle into meat with the control of the inhibitor. However, the existence of MBSP and its endogenous inhibitor in ostrich skeletal muscle has not been reported. Purification of MBSP and its endogenous inhibitor to homogeneity is a prerequisite for studying their mechanism of action in the skeletal muscle of organisms. The mechanism of action of MBSP and MBSPI has been a subject of interest to researchers for many years. However, the difficulty to dissociate such an enzyme from myofibrils and its scarcity in muscle tissues of the various fish species studied and mouse have been reported (Cao *et al*., 2006a).

Studies have been conducted in our laboratory as part of a systematic investigation of the biochemistry of serine proteases and their precursors, and inhibitors in the ostrich: trypsinogen (Bodley *et al*., 1995; Smith *et al*., 1992; 1993a;Szenthe *et al.,* 2005), trypsin (Hartley *et al*., 1987), chymotrypsins (Smith *et al.,* 1992), enterokinase (Naude *et al*., 1993), α_1-proteinase serum inhibitor (Kuhn *et al*. 1994), α_1-antichymotrypsin-like serum inhibitor (Frost *et al*., 1997), α_2-macroglobulin serum inhibitor (Van Jaarsveld *et al.,* 1994), serum α_2-antiplasmin and plasmin/plasminogen (Thomas *et al.,* 2001), pancreatic secretory trypsin inhibitor (Zhao *et al*., 1996), thrombin and antithrombin (Frost *et al.,* 2000, 2002) and proteasome (Klinkradt *et al.,* 1997, Thomas *et al*., 2002, 2004a, 2005).

The objectives of the present study were:

- To investigate the possible existence of ostrich MBSP and its endogenous inhibitor in ostrich skeletal muscle.
- To isolate and purify ostrich MBSP and its endogenous inhibitor from ostrich skeletal muscle.
- To determine the activity of MBSP and its endogenous inhibitor in ostrich muscle.

- To partially characterize ostrich MBSP and its endogenous inhibitor physicochemically and kinetically.
- To analyze the N-terminal amino acid sequence of ostrich MBSP and MBSPI.

CHAPTER 3: MATERIALS AND METHODS

3.1 Protein determinations

Many methods for estimating protein concentration are well documented (Stoscheck, 1990; Rehm, 2006) and the appropriate choice of a method depends on five major criteria: (1) the amount of protein available to assay, (2) the concentration of the protein, (3) the specificity of the assay, (4) the presence of chemicals that may interfere with the assay and (5) the ease and reliability of performing the assay. In addition, an approximate range of sensitivity is given for each assay (Lowry *et al.*, 1951; Bradford, 1976; Smith *et al.,* 1985; Starcher, 2001).

3.1.1 Spectrophotometric assay

Proteins absorb light in the ultraviolet region with 2 maxima, λ_{280nm} and λ_{210nm}. Absorption spectroscopy involves the absorption of a photon by an electron (Stoscheck, 1990).

3.1.2 BCA assay

3.1.2.1 Introduction

The bicinchoninic acid (BCA) assay was used to determine protein content after each step of the isolation and purification of MBSP and MBSPI according to Smith *et al*. (1985). BCA is a stable, water-soluble compound capable of forming an intense purple complex with cuprous ions in an alkaline solution.

The reagent forms the basis of an analytical method capable of monitoring cuprous ions produced in the reaction of a protein with an alkaline solution (Biuret reaction). The colour produced from this reaction is stable and increases in a proportional fashion over a broad range of increasing protein concentrations. BCA has a good tolerance towards commonly encountered interferences such as nonionic detergents and buffer salts. The reagent stability and resulting chromophore allow for a simplified assay, leading to a one-step analysis and an enhanced flexibility in protocol selection. The assay has a high

sensitivity towards protein concentration that ranges from 0.5 to 1mg/ml and results in low protein-to-protein variations associated with other protein determination assays (Smith *et al*., 1985).

3.1.2.2 Reagents

For the BCA assay, two reagents were required, prepared and stored in a dark bottle:

Reagent A consists of an aqueous solution of 1% (w/v) BCA-Na$_2$ (Sigma), 2% (w/v) Na$_2$Co$_3$, 0.16% (w/v) K tartrate, 0.4% (w/v) NaOH, and 0.95% (w/v) NaHCO$_3$ in ddH$_2$O. Potassium tartrate was used, as Na tartrate was not available.

Reagent B consists of 4% (w/v) CuSO$_4$.5H$_2$O in ddH$_2$O.

Reagents A and B are stable indefinitely at room temperature.

Standard Working Reagent (S-WR) was prepared freshly by mixing 50 vol. (8.5 ml) of Reagent A with 1 vol. (170 µl) of Reagent B.

3.1.2.3 Procedure

Bovine serum albumin (BSA) is generally used as a protein standard because it is widely available in high purity and is relatively inexpensive. Test samples were diluted to give three dilutions prior to protein assay and each was assayed in triplicate.

The BCA standard assay procedure was carried out in a microtiter plate (NUNC^{TM} Apogent, Denmark) in which 200 µl of S-WR was added to 10 µl of sample, BSA standards and blank in triplicate. The mixture was shaken and incubated at 37˚C for 30 min using the Fluoroskan Ascent FL ThermoLabsystem microtiter plate incubator. The absorbance was read at λ_{570nm} with a Bio-Tek KC4 power wave XS microtiter plate reader. The sample buffer was used as a blank. A BCA protein concentration calibration curve is shown in Figure 3.1.
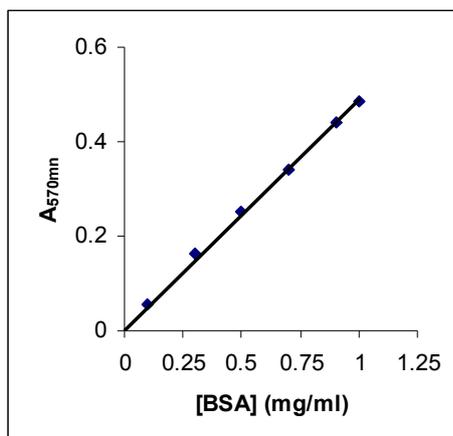
Figure 3.1. BCA protein calibration curve ($r^2 = 0.9997$).

3.2 Enzymatic activity assay

The MBSP activity assay using both synthetic and native protein substrates were reported (Sangorrin *et al*., 2000; Ohkubo *et al.,* 2004a). Commercial bovine trypsin (Worthington Biochemical cooperation) was used as a positive control for the assay. Boc-Phe-Ser-Arg-MCA was obtained from Sigma. Black microtiter plates were purchased from NUNC.

The trypsin activity assay using various bovine trypsin concentrations was prepared in triplicate as per MBSP enzyme activity assay. The linear relationship between the rate of hydrolysis of the substrate and the enzyme concentration is shown in Figure 3.2.
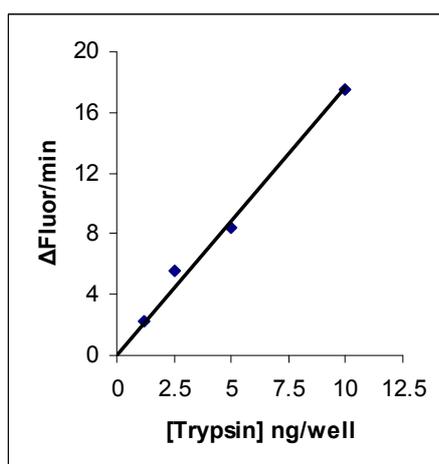
Figure 3.2. Commercial bovine trypsin activity calibration curve ($r^2 = 0.953$).

3.2.1 MBSP and trypsin activity assay

The MBSP assay was carried out according to Ohkubo *et al.* (2004a). MBSP activity was performed in triplicate using Boc-Phe-Ser-Arg-MCA, which is a fluorogenic substrate. Twenty (20) µl 50 µM Boc-Phe-Ser-Arg-MCA in 50 mM Tris-HCl buffer (pH 8) was added to 20 µl of enzyme solution in a black microtiter plate. Sample buffer was used as a blank. After incubation for 10 min at 40˚C, the reaction was terminated by addition of 180 µl stopping solution (dH$_2$O: MeOH: n-butanol; 35:30:35; v/v/v). The fluorescence intensity of the liberated MCA was measured using a Fluoroskan Ascent FL spectrophotometer at Exλ_{355nm} and Emλ_{460nm}. Enzyme activity was defined as a change in fluorescence per min (ΔFluor/min).

3.2.2 MBSP and bovine trypsin inhibitory activity assays

The inhibition assay was carried out according to Sangorrin *et al.* (2000) and Cao *et al.* (2001). Bovine trypsin was used as the source of the enzyme for the inhibition assay.

Prior to incubation, 40 µl of the trypsin inhibitor solution or sample buffer was added to 40 µl of a bovine trypsin solution containing the same protein content, e.g. 3.123 µg: 3.125 µg; E:I. After 30 min incubation at 40˚C in a shaking water bath to allow enzyme to interact with the inhibitor, a 20 µl aliquot was added to 20 µl 50 µM Boc-Phe-Ser-Arg-MCA in 50 mM Tris-HCl buffer (pH 8) in triplicate in a black microtiter plate. Thereafter, the enzyme activity assay was carried out as described in section 3.2.1. Inhibitory activity was expressed as a percentage compared to a trypsin control treated in the same way as the test solutions.

3.3 Electrophoretic techniques

3.3.1 SDS-PAGE

3.3.1.1 Introduction

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is an excellent method to identify and monitor proteins during purification and it analyses the

purity of separated fractions. SDS-PAGE is also routinely used to determine the M_r of purified fractions. SDS-PAGE can be scaled up and used in a preparative mode to yield sufficient protein for further studies. However, two-dimensional analysis, combining isoelectric focusing with SDS-PAGE, is a high-resolution method for protein fractionation, enabling many polypeptides to be resolved in a single gel. In addition, when used in conjunction with blotting methods, SDS-PAGE provides one of the most powerful means available for protein analysis (Garfin, 1990).

Gel electrophoresis is generally based on the sizes, shapes and net charges of macromolecules. SDS-PAGE overcomes the limitation of native PAGE by imposing uniform hydrodynamic and charge characteristics on all the proteins in a sample mixture. During sample preparation, proteins are treated with SDS in a ratio of 1:1 (w/w), imparting a negative charge to the resultant complexes. Interaction of the sample with SDS disrupts all non-covalent bonds by causing the macromolecules to unfold. Sample treatment with a disulfide reducing agent, such as ß-mercaptoethanol or dithiothreitol, further denatures proteins, breaking them down to their constituent subunits. The electrophoretic mobilities of the resultant detergent-polypeptide complexes all assume the same functional relationship to their molecular weights. Migration of SDS derivatives is towards the anode at a rate inversely promotional to the logarithms of their molecular weights. Molecules with low molecular weight migrate faster than those with high molecular weight. (Garfin, 1990). SDS-PAGE was performed according to Laemmli (1970) with some modification. A vertical minigel system (Bio-Rad) with an 8x7 cm cell format was used during the course of this study to estimate the M_r of proteins of interest.

3.3.1.2 Reagents and materials

Tris, TEMED, APS, 40% acrylamide/bis stock, Coomassie Brilliant Blue R-250 (CBB), glycerol and SDS were purchased from Bio-Rad Laboratories.

3.3.1.3 Procedure

The electrophoretic procedure was carried out as described in the Bio-Rad instruction manual. The stacking and resolving gels were prepared as shown in Table 3.1.

Table 3.1. Preparation of staking and resolving gels (2 gels).

Components	Stacking gel	Resolving gel	
	4%	12.5 %	13.5 %
dH$_2$O	3.213 ml	4.225 ml	3.97 ml
Solution A (40%, w/v, acrylamide/bis stock solution)	0.488 ml	3.126 ml	3.38 ml
Solution B (1.5 M Tris-HCl buffer, pH 8.8)	-	2.5 ml	2.5 ml
Solution C (0.5 M Tris-HCl buffer, pH 6.8)	1.25 ml	-	-
Solution D (10%, w/v, SDS)	50 µl	100 µl	100 µl
TEMED	5 µl	5 µl	5 µl
APS	25 µl	50 µl	50 µl

Samples were reduced by placing in a boiling water bath for 3 min in the presence of 5% (v/v) ß-mercaptoethanol. Electrophoresis was performed at 100V and 40mA for ~2 h at room temperature.

Low molecular weight markers (LMW) (14.2 − 66 kDa) were used and a calibration curve is shown in Figure 3.3.
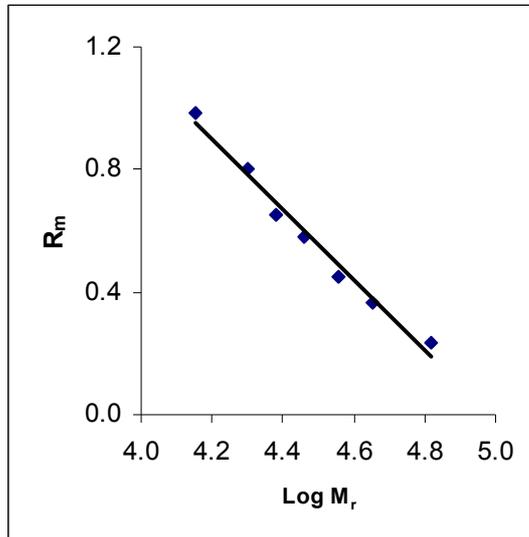
Figure 3.3. Calibration curve using SDS-PAGE (12.5% gel). M_r markers: 66 kDa, albumin; 45 kDa, ovalbumin; 36 kDa, glyceraldehyde-3-phosphate dehydrogenase; 29 kDa, carbonic anhyhrase; 24 kDa, trypsinogen; 20 kDa, trypsin inhibitor and 14.2 kDa, lactalbumin (r^2= 0.982).

3.4 Zymography

3.4.1 Introduction

Zymography is an electrophoretic method for measuring proteolytic activity. It is simple, sensitive, quantifiable and a functional assay for analyzing proteolytic activity. Zymography offers several features which render it particularly useful with respect to alternative methods such as ELISA: no expensive materials are routinely required (e.g. antibodies) and several proteases showing activity on the same substrate can be detected and quantified on a single gel. The standard method is based on SDS-PAGE with the gel impregnated with a protein substrate, in particular casein or gelatin, which is degraded by the proteases, resolved during the incubation period. CBB stain of the gel reveals sites of proteolysis as white bands on a dark blue background. This leads to fast and reproducible

staining of zymograms permitting reliable quantitation of proteolytic activity (Leber and Balkwill, 1997).

3.4.1.1 Ostrich MBSP zymography

The caseinolytic activity of partially purified ostrich MBSP fractions was detected using casein-SDS-PAGE according to Venugopal and Saramma (2006). SDS-PAGE (12.5%, v/v) was polymerized with casein to get a final concentration of 1% (w/v) casein. The samples were applied in non-reducing sample buffer without denaturation and electrophoresis was performed as described in section 3.3.1.3. Following the electrophoresis, the gels were washed twice (15 min each) with 2.5% (v/v) Triton-X 100 to remove SDS and was incubated overnight in 50 mM Tris-HCl buffer (pH 8) containing 0.1 M NaCl at 37°C in a water bath. The gels were stained with 0.5% (w/v) CBB. The caseinolytic activity was revealed as a clear zone, which was visualized by destaining the gels with the destain solution (methanol: acetic acid: water, 50:10:40, v/v/v).

3.4.1.2 Ostrich MBSPI reverse zymography

The anti-trypsin caseinolytic activity of partially purified ostrich MBSPI fractions was performed as described in section 3.4.1 with a minor change. Following the removal of SDS from gels by 2.5% (v/v) Triton-X 100, the gels were incubated overnight in 10% (w/v) commercial bovine trypsin in 50 mM Tris-HCl buffer (pH 8) containing 0.1 M NaCl at 37°C in a water bath. The gels were stained with 0.5% (w/v) CBB. The anti-trypsin band was visualized by destaining the gels with the destain solution.

3.5 Western blot

3.5.1 Introduction

A Western blot is a method to detect a specific protein in a given sample, tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the size of the polypeptide under denaturing or non-denaturing conditions, followed by transfer of proteins to a membrane (Burnette, 1981).

3.5.2 Semi-dry protein transfer

3.5.2.1 Procedure

SDS-PAGE was performed as described in the Bio-Rad instruction manual, followed by the transfer procedure as described in section 3.3.1.3. The proteins were transferred for 2 h at 25 V, using a current limit of 2 mA. After the transfer, the PVDF membranes were stained for 15 min with CBB R-250 to detect protein bands, followed by destaining. After brief destaining, the PVDF (nitrocellulose) membranes were air dried and the blots were sent to Prof. Koji Muramoto (Sendai, Japan). The gels were also stained and destained to check that the transfer was successful.

3.6 N-terminal sequence analysis

The N-terminal amino acid sequence analysis of bands from a PVDF membrane was performed by Prof. Muramoto by automatic sequencing with a Shimadzu PSQ-1 gas-phase protein sequencer using the PITC coupling method (Muramoto *et al.*, 1993).

3.7 Column chromatographic procedures

3.7.1 Introduction

Chromatography is the most accepted separation tool in modern Biochemistry laboratories. Each chromatographic method exploits different physical or biological properties of the molecule as a basis of separation (Kennedy, 1990). With almost all chromatographic materials, column chromatography is superior to the batch method. Specially developed matrix materials that are packed in glass, stainless steel, or polyether ketone columns permit high flow rates at medium pressures. Resins are available for gel adsorption, ion exchange, hydrophobic interaction and affinity chromatographies (Rehm, 2006).

3.7.2 Ion-exchange chromatography

In ion exchange chromatography (IEC), proteins bind to a matrix via electrostatic interactions. The matrix carries positively charged groups (anion exchanger) or negatively charged groups (cation exchanger). Extent and strength of the binding of a protein to the ion exchanger depend on the pH and ionic strength of the buffer solution, the isoelectric point of the protein, and the density of the charges on the matrix (Rehm, 2006). IEC involves two separate events: (1) the binding of the protein to the fixed charges and (2) the elution or displacement of the protein from the fixed charges. The ability of counter ions (salts) to displace proteins bound to fixed charges is a function of the difference in affinities between the fixed charges and the nonfixed charges of both the protein and salts. Affinities in turn are affected by several variables, including the magnitude of the net charge of the protein and the concentration and type of salts used for displacement (Rossomando, 1990).

In addition, IEC yields good purification factors of between 3 and 15, depending on the protein and elution conditions, and it concentrates the sample. The yield is also good, between 50 and 80%, compared to gel exclusion and hydrophobic interaction chromatography. Alternatively, with multistep purifications, it is a good idea to use IEC at the beginning of a purification procedure. The separating capacity of IEC also depends on the flow rate; the slower the flow rate the better the resolution. With IEC of membrane proteins, charged detergents such as cholate or deoxycholate are to be avoided (Rehm, 2006). In this study, the anion exchange resins used were Toyopearl Super Q 650S and prepacked columns of HiTrap Q HP, as well as cation exchange prepacked columns of HiTrap SP HP.

3.7.2.1 Regeneration of IEC resins

3.7.2.1.1 Toyopearl Super Q 650S

Toyopearl Super Q 650S resin was purchased from TOSOH Company, Japan (gift from Prof. Muramoto). Toyopearl Super Q 650S resin was washed three times with dH$_2$O to remove fines prior to regeneration. The washed resin was regenerated and equilibrated as follows:

1. Wash with 5 bed volumes (v/v) of 0.5 M HCl for 30 min and immediately decant the HCl.

2. Wash with 10 bed volumes (v/v) of dH$_2$O for 40 min and decant (repeat 3 times).

3. Wash with 5 bed volumes (v/v) of 0.5 M NaOH for 30 min and immediately decant.

4. Repeat step 2.

5. Equilibrate with 5-10 bed volumes (v/v) of starting buffer. Repeat, checking the pH of the wash buffer, until it is equivalent to that of the starting buffer or if resin is not ready to be used, is stored in dH$_2$O containing a few drops of 0.02% (w/v) sodium azide.

3.7.2.1.2 HiTrap Q HP and HiTrap SP HP

HiTrap SP HP (5ml) and HiTrap Q HP (5ml) are prepacked columns for operation with a Fast Protein Liquid Chromatography (FPLC) system. The operation, cleaning and storage of these columns were followed as outlined in the Amersham Biosciences instruction manual. Up to five columns were connected in series and used for sample separation in this study.

3.7.3 Size exclusion chromatography

Size exclusion chromatography (SEC) or gel filtration is based on the different distribution of molecules between a gel compartment and the surrounding media (Rehm, 2006). SEC is performed using porous beads as the chromatographic support. Discrimination between molecular species on the basis of size is based on differential

permeation into matrices of controlled porosity. It is commonly accepted that small molecules, such as deuterium oxide, totally permeate the liquid volume within and between the particles in a column and elute from the column in a volume of liquid known as the total permeation volume (V_t). In contrast, macromolecular species that are too large to penetrate the pore matrix of the column packing elute in a column void volume (V_0), equal to the volume of liquid between particles. Resolution in SEC is determined by three characteristics of the chromatographic packing: (1) pore size distribution, (2) pore volume and (3) particle size. A small pore size distribution gives the greatest discrimination between solutes based on molecular size. Uniformity of pore distribution can be seen in the slope of log M_r versus V_e curve, where V_e is the elution volume of all macromolecular species (Chicz and Regnier, 1990). Sephadex G-75 and Superdex 200 HR were used in this study.

3.7.3.1 Preparation of SEC gel

3.7.3.1.1 Sephadex G-75

A slurry was prepared according to the procedure of the manufacturer (Pharmacia Biotech). After swelling 50 g of dried Sephadex G-75 powder (medium to fine), the slurry was washed 3 times with dH_2O and then washed several times with the equilibration buffer until the pH of the equilibration buffer was reached. Free sites on the gel (ionic in nature) were coated by stirring BSA (2 g solubilised in buffer) into the gel for 2 h. The unbound proteins were poured off and the bound protein was eluted with 1.6 l 0.5 M NaCl in the equilibration buffer. The gel was washed several times with 5 volumes of dH_2O and protein was monitored at A_{280nm} until it reached a baseline value. The gel was washed several times with the equilibration buffer and allowed to stand overnight at 4°C before being packed into a column.

3.7.3.1.2 Superdex 200 HR

Superdex 200 HR is available in a prepacked column for operation with a FPLC system. The operation, cleaning and storage of this column were followed as outlined in the Amersham Biosciences instruction manual.

3.7.4 Hydrophobic interaction chromatography

Hydrophobic interactions are a phenomenon of great biological significance. Hydrophobic interactions are involved in antigen-antibody and enzyme-substrate reactions (Kennedy, 1990). Many soluble proteins have hydrophobic areas on the surface. In watery solution, these hydrophobic areas associate with hydrophobic surfaces. The inclination towards association depends on the structure of the water, and this in turn from the salt dissolved in it. In addition, high concentrations of certain ions increase the hydrophobic interactions, whereas chaotropic salts disrupt the water structure and thereby reducing the inclination for hydrophobic interactions. Hydrophobic interaction chromatography (HIC) takes advantage of these conditions. It is compatible with ammonium sulfate precipitation. The protein sample is loaded with high ionic strength onto a hydrophobic matrix. The protein binds, the column is washed and the sought-after protein is eluted with a decreasing salt gradient or a lower salt concentration. Detergents that cover the hydrophobic areas of the proteins and thereby remove them from the matrix can also be used. Matrices for HIC are derivatized with either phenyl or octyl residues (Rehm, 2006).

3.7.4.1 Regeneration of HIC resin

3.7.4.1.1 Phenyl Sepharose CL-4B

Phenyl Sepharose CL-4B was purchased from Sigma and regenerated according to Kennedy (1990) as follows:

1. Wash with 1 bed volume (v/v) dH_2O for 30 min and decant.

2. Wash with 1 bed volume of 25, 50 and 95% (v/v) ethanol for 30 min each and decant.

3. Wash with 2 bed volumes of n-butanol for 30 min and decant.

4. Wash with 1 bed volume of 95, 59, and 25% (v/v) ethanol for 30 min each and decant.

5. Repeat step 1 (three times).

6. Equilibrate the gel with the starting buffer (repeat, checking the pH of the wash buffer until it is equivalent to that of the starting buffer), or if resin is not ready to be used, is stored in dH_2O containing a few drops of 0.02% (w/v) sodium azide.

3.7.5 Affinity chromatography

Affinity chromatography is one of the most powerful methods that can be applied to enzyme purification (Ostrove, 1990). Affinity chromatography exploits the specific binding of a protein for another molecule, its ligand (e.g. enzyme). The ligand is immobilized on an insoluble matrix, which is packed into a column (Hames and Hooper, 2005). A good matrix for affinity chromatography should have the following properties: (1) hydrophilic in nature, reduce nonspecific interactions; (2) large pores to allow all areas of the matrix to be available to most of the molecular mixture; (3) rigid structure, the matrix must withstand the pressures of packing and solvent flow during elution or washing; (4) inert in nature, the matrix should not contribute to the separation, and (5) chemical stability, the matrix must be stable to all solvents used in the separation (Ostrove, 1990). A commonly employed combination of immobilized ligand and protein to be used in affinity chromatographic systems, include an inhibitor to purify an enzyme, an antibody to purify its antigen, a hormone (e.g. insulin) to purify its receptor and lectin (e.g. concanavalin A) to purify a glycoprotein. On adding a mixture of proteins onto a packed column, only the protein of interest binds to the ligand. All other proteins pass straight through the column. The bound protein is eluted from the immobilized ligand in a highly purified form (Hames and Hooper, 2005).

3.7.5.1 Preparation of affinity resins

3.7.5.1.1 L-Arginine-Sepharose CL-4B resin

The L-arginine-Sepharose CL-4B resin was prepared according to Thomas (2004b) with some modification. All reagents were freshly prepared and all steps were carried out using two fume hoods, one for weighing cyanogen bromide (CNBr) and the other for carrying out the different reactions, as outlined in Diagram 3.1.

Diagram 3.1: Procedure for the preparation of L-arginine-Sepharose CL-4B resin.

```
┌─────────────────────────────────┐
│   25ml Sepharose CL-4B resin    │
└─────────────────────────────────┘
                │
```

1. Wash resin with 16 volumes dH_2O.
2. Suspend resin in 25 ml dH_2O.
3. Dissolve 2.5 g CNBr in 2.5 ml 300 mM 1,4-dioxane and add to suspension.
4. Immediately raise pH to 11 with 500 mM NaOH for 9 min and maintain temperature at 20˚C by adding small amounts of ice.
5. Stop the reaction by adding large amount of ice.
6. Transfer the suspension to a Büchner funnel and wash under suction with 20 volumes of ice cold dH_2O.

```
┌─────────────────────────────────┐
│      Sepharose CL-4B resin      │
│          (activated)            │
└─────────────────────────────────┘
                │
```

7. Suspend in 2 volumes 100 mM $NaHCO_3$ buffer (pH 9.5).
8. Dissolve 11 g 1,6-diaminohexane in 25 ml 100 mM $NaHCO_3$ buffer (pH9.5) and 25 ml N-N-dimethylformamide.
9. Mix with resin and stir overnight at room temperature.

```
┌─────────────────────────────────┐
│     Diaminohexane-Sepharose     │
│          CL-4B resin            │
└─────────────────────────────────┘
                │
```

10. Dissolve 2.5 g L-arginine in 25 ml 10 mM NaOH.
11. Mix with resin, heat at 60˚C and allow to cool overnight at room temperature.
12. Wash with 500 ml dH_2O, 250 ml 100 mM ethanol, 250 ml 500 mM NaCl and 2 L dH_2O.

```
┌─────────────────────────────────┐
│   L –arginine Sepharose CL-4B   │
│             resin               │
└─────────────────────────────────┘
```

3.7.5.1.2 Trypsin-Sepharose CL-4B affinity resin

Trypsin-Sepharose CL-4B affinity resin was prepared according to Smith (1993b) and the Sigma instruction manual as outlined in Diagram 3.2.

Diagram 3.2: Procedure for the preparation of trypsin-Sepharose CL-4B resin.

```
┌─────────────────────────────────┐
│   50 ml Sepharose CL-4B resin   │
└─────────────────────────────────┘
```

1. Wash resin with 10 volumes dH_2O.
2. Add 50 ml dH_2O.
3. Dissolve 5 g CNBr in 5 ml 300 mM 1, 4-dioxane.
4. Add CNBr solution to suspension, raise pH to 11 and maintain with 500 mM NaOH for 12 min.
5. Maintain temperature at 20˚C by adding small amounts of ice.
6. Stop reaction by adding a large amount of ice.
7. Transfer to a Büchner funnel and wash under suction with 10 volumes of ice-cold coupling buffer [100 mM $NaHCO_3$ buffer (pH 8.5) containing 500 mM NaCl].

```
┌─────────────────────────────────┐
│      Sepharose CL-4B resin      │
│          (activated)            │
└─────────────────────────────────┘
```

8. Dissolve 150 mg bovine trypsin in 50 ml of ice-cold coupling buffer.
9. Mix with resin and gently shake overnight at 4˚C.
10. Wash with 20 volumes coupling buffer.
11. Add the suspension to 200 ml blocking buffer [100 mM ethanolamine buffer (pH 8)] and stir overnight at 4˚C.
12. Wash with (1) ice cold coupling buffer and (2) ice cold Na acetate buffer (pH 4). Repeat step 12 three times.
13. Equilibrate with equilibration buffer or store the resin in 1.0 M NaCl at 2-8˚C with sodium azide.

```
┌─────────────────────────────────┐
│    Trypsin-Sepharose CL-4B      │
│             resin               │
└─────────────────────────────────┘
```

3.7.5.1.3 *p*-Aminobenzamidine-Agarose resin

p-Aminobenzamidine-Agarose resin (10 ml) was purchased from Sigma. The generation and storage of the resin were followed as outlined in the Sigma instruction manual.

3.7.6 Hydroxylapatite chromatography

Hydroxylapatite (HA) is a crystalline form of calcium phosphate. The adsorption of proteins to HA is due to (1) the primary non-specific electrostatic interactions between the positively charged amino groups of proteins and the general negative charges on the HA resin when equilibrated with phosphate buffers, (2) the electrostatic repulsion of the carboxyl groups of proteins due to the negative charge of the resin and (3) the specific

binding by complexation of the carboxyl groups of proteins to the calcium sites on the resin (Gorbunoff, 1990).

The HA resin was regenerated by washing with a high salt concentration (0.5 M borate phosphate buffer (pH 7.5) to remove all tightly bound materials and then with repeated volumes of dH$_2$O. It was equilibrated with 1mM borate phosphate buffer (pH 7.5).

3.8. Kinetic characterization of ostrich MBSP and commercial bovine trypsin

3.8.1 Substrate specificity

Substrate specificities of ostrich MBSP and commercial bovine trypsin were studied using various synthetic substrates (10 μM). Appropriate blanks were used as controls. Relative enzyme activity was estimated based on the activity with a specific synthetic trypsin substrate Boc-Phe-Ser-Arg-MCA taken as 100%.

3.8.2 pH optimum

The effect of pH on purified ostrich MBSP and commercial bovine trypsin was studied by measuring activities at various pH values, using 100 μM Boc-Ser-Arg-MCA (dissolved in dH$_2$O, instead of 0.1 M Tris-HCl buffer, pH 8.0) at 40˚C as substrate. pH was varied from 2-10 using the single buffer system (100 mM Tris/50 mM histidine/100 mM sodium acetate) and 20 μl enzyme solution was mixed with 10 μl buffer solution and 10 μl substrate. The exact pH value of the mixture was evaluated by measuring the pH with a microelectrode after adding all assay components. Relative enzyme activity was estimated based on the highest activity taken as 100%.

3.8.3 Temperature optimum

Optimum temperature of ostrich MBSP and commercial bovine trypsin was studied by measuring activities at pH 8.0 using 50 μM Boc-Phe-Ser-Arg-MCA in 100 mM Tris/50 mM histidine/100 mM sodium acetate buffer system and varying temperatures. The assay solution was equilibrated for 5 min at an appropriate temperature in a LAUDA circulating water bath before starting the reaction by addition of an enzyme solution and

incubation at that appropriate temperature. Relative enzyme activity was estimated based on the highest activity taken as 100%.

3.8.4 Kinetic parameters

The assay was performed as described in sections 3.2.1 and 3.2.2, with some minor changes. The kinetic parameters of ostrich MBSP and commercial bovine trypsin were studied using one synthetic substrate, Boc-Val-Pro-Arg-MCA, in 50 mM Tris-HCl (pH 8.0), at various concentrations (1.5625, 3.125, 6.25, 12.5, 25, 50, and 100 μM). The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) values were estimated by constructing Michaelis-Menten and Lineweaver-Burk plots.

CHAPTER 4: ISOLATION AND PURIFICATION OF OSTRICH MBSP AND ITS ENDOGENOUS INHIBITOR

4.1 ISOLATION AND PURIFICATION OF OSTRICH MBSP

4.1.1 FIRST ISOLATION AND PURIFICATION ATTEMPT

4.1.1.1 Isolation procedure

Ostrich MBSP was isolated from skeletal muscle according to Ohkubo *et al.* (2004a). All the steps were carried out at 4°C unless stated otherwise. Fresh ostrich skeletal muscle (930 g) was trimmed of fat and minced with a meat mincer. The minced meat (600 g) was mixed with 4 volumes of 10 mM borate buffer ($Na_2B_4O_7$-KH_2PO_4) (pH 7.5) and homogenized using a Waring blender at low speed for 30 s (3x) with 10 s intervals. The homogenate was centrifuged at 10000xg for 20 min. The pellet (myofibrillar fraction) was rehomogenized in 4 volumes of ice-cold dH_2O, adjusted to pH 6 with 1 M HCl and centrifuged at 5000xg for 15 min. The myofibrillar fraction was washed 3 times sequentially with 4 volumes of ice cold dH_2O by centrifugation as described above. Washing of the myofibrillar fraction was repeated to elute sarcoplasmic enzymes from myofibrils. The resulting washed myofibrillar fraction was homogenized for 5 s and adjusted to pH 6 with 1 M HCl.

The homogenate was heated in a water bath at 78°C for 1 h with gentle stirring using an overhead mechanical stirrer until the homogenate reached 55°C, and kept at the same temperature for 10 min. The heat extract was immediately cooled to 4°C and allowed to stand overnight. The extract was centrifuged at 10000xg for 10 min and the supernatant was collected as the crude MBSP extract, adjusted to pH 6 with 1M HCl and kept at 4°C for 3 days. The crude MBSP extract was adjusted to pH 7 with 1 M NaOH and lyophilized. The lyophilized powder (12.215 g) was dissolved in 50 ml 1 M KCl by gently stirring for 2 h, adjusted to pH 5 and allowed to stand overnight at 4°C. The KCl extract was centrifuged at 12000xg for 10 min. The supernatant (48 ml) was adjusted to pH 7 with 3 M NaOH and dialyzed overnight against basal buffer (10 mM $Na_2B_4O_7$-

KH_2PO_4, pH 7.5). The dialyzed enzyme solution was centrifuged at 5000xg for 15 min. The supernatant (65 ml) was treated with cold ethanol (-30°C) and centrifuged at 20000xg for 20 min at -10°C. The supernatant was dialyzed overnight against basal buffer, concentrated with PEG 20000 and dialyzed against the same buffer.

4.1.1.2 Column chromatography purification steps

Crude ostrich MBSP (fraction Q) was purified by two chromatographic steps, namely Toyopearl Super Q 650S, followed by hydroxylapatite chromatography.

4.1.1.2.1 Toyopearl Super Q 650S chromatography

Toyopearl Super Q 650S resin was regenerated and equilibrated as outlined in section 3.7.2.1.1. The dialyzed crude enzyme solution (85 ml) obtained in section 4.1.1.1 was applied to a Toyopearl Super Q column (1.6 x 25 cm) equilibrated with equilibration buffer (20 mM Tris-HCl, pH 7.5) at 18 ml/h. Unadsorbed proteins were eluted with the same buffer until A_{280nm} reached a baseline value. The adsorbed proteins were eluted by a linear salt gradient from 0 to1000 mM NaCl (total volume 200 ml) in the same buffer at 25 ml/h. Pooled fractions were concentrated with PEG 20000, dialyzed against 10 mM $Na_2B_4O_7$-KH_2PO_4 buffer (pH 7.5) and assayed for enzyme activity. The fractions were stored in 50% ethylene glycol and kept at -20°C until used.

4.1.1.2.2 Hydroxylapatite chromatography

Hydroxylapatite resin was regenerated and equilibrated as outlined in section 3.7.6. Fraction Q3 obtained from section 4.1.1.2.1 was applied to a hydroxylapatite column (1.6 x 11.5 cm) equilibrated with 1 mM borate-phosphate buffer (pH 7.5) at 5 ml/h. Unadsorbed proteins were eluted with the same buffer until A_{280nm} reached a baseline value. The adsorbed proteins were eluted by a linear salt gradient from 1 to 200 mM borate-phosphate buffer at 20 ml/h. Pooled fractions were assayed for enzyme activity and lyophilized.

4.1.1.3 Results and discussion

In Figure 4.1 the breakthrough peak could not be shown in the elution profile, due to fraction collector problems during fractionation, thus leading to a loss of unadsorbed fractions.
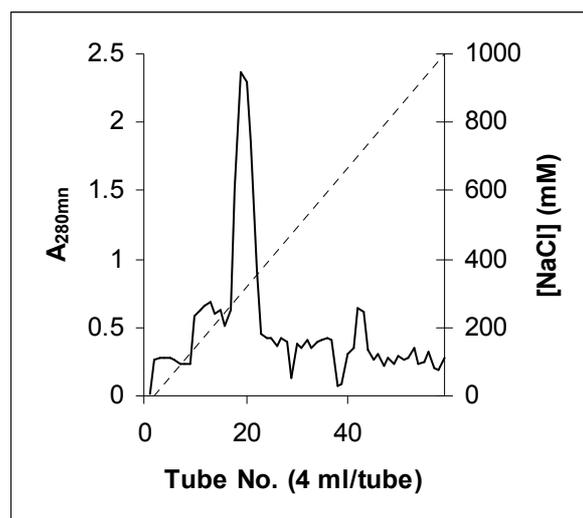
Figure 4.1. Toyopearl Super Q 650S chromatography of heat- and alcohol-treated extract (fraction Q) (789 mg). Column: 1.6 x 25 cm; Linear salt gradient: 0-1000 mM NaCl in borate-phosphate buffer (pH 7.5). Tubes were pooled: 2-9 (Q1); 10-17 (Q2); 18-22 (Q3); 23-28 (Q4); 29-37 (Q5); 38-39 (Q6); 40-41 (Q7); 42-43 (Q8); 44-56 (Q9). $-A_{280nm}$; --- [NaCl].

Tubes were pooled (Figure 4.1), and fraction Q3, showing MBSP activity underwent hydroxylapatite chromatography. The hydroxylapatite column was found to be blocked easily because of buffer salt precipitation when buffer was passed through the column; therefore, fraction Q3 was loaded slowly. The elution profile of hydroxylapatite chromatography is shown in Figure 4.2.
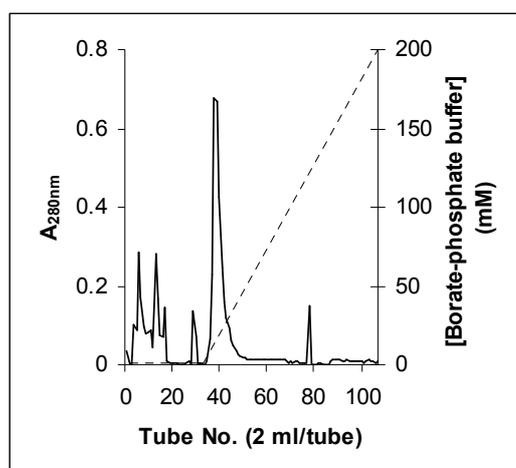
Figure 4.2. Hydroxylapatite chromatography of fraction Q3 (358.7mg). Column: 1.6 x 11.5 cm. Linear salt gradient: 1-200 mM borate-phosphate buffer (pH 7.5). Tubes were pooled: 2-19 (HA1); 37-51 (HA2). ─A$_{280nm}$; --- [borate-phosphate buffer]

A summary of isolation and purification of the first attempt is shown in Table 4.1.

Table 4.1. Summary of the first isolation and purification of the first attempt of ostrich MBSP from 600 g minced skeletal muscle.

Purification step	Fraction code	Volume (ml)	Total protein (mg)	Total activity (U)*	Specific activity (U/mg)*	Purification (fold)	Yield (%)
Alcohol-treated extract	Q	85	789	615.0	0.8	1	100
Toyopearl - Super Q 650S	Q3	25	358.7	102.5	0.3	0.4	16.7
Hydroxylapa-tite	Q3HA2	22	48	541	11.2	14.1	88

* U= ΔFluorescence/min

The SDS-PAGE patterns of the lyophilized fraction Q3HA2 after hydroxylapatite chromatography are shown in Figure 4.3.
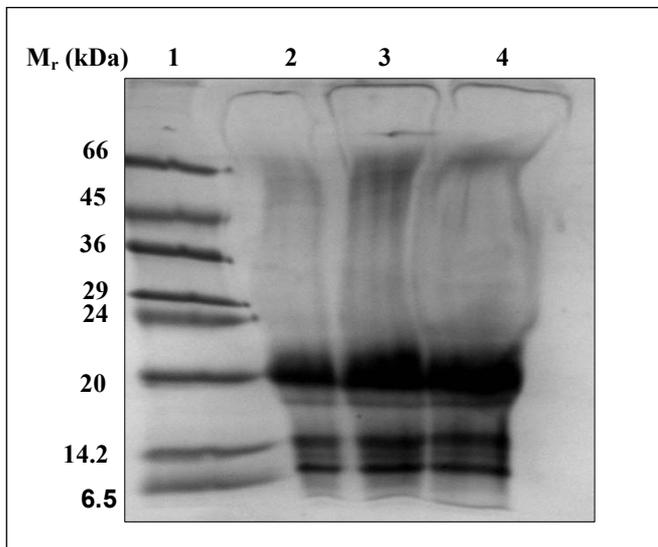
Figure 4.3. SDS-PAGE patterns of fraction Q3HA2 resulting from hydroxylapatite chromatography under reducing conditions. Lanes: 1, LMW markers; 2, 30 μg; 3, 60 μg; 4, 120 μg.

Ostrich MBSP was isolated from washed myofibrils (pH 6) by heat treatment at 55°C for 10 min, acid treatment at pH 6, before and after lyophilization, and alcohol treatment, followed by two chromatographies on Toyopearl Super Q 650S and hydroxylapatite. The heat-treatment denatured some contaminating myofibrillar proteins and dissociated the target enzyme from myofibrils (Cao *et al.,* 2006a; Ohkubo *et al.,* 2004a). The purification results of MBSP are summarized in Table 4.1. Approximately 48 mg of partially purified ostrich MBSP was obtained from 600 g of skeletal muscle and the specific activity increased 14.1-fold with a recovery of 88% after purification on hydroxylapatite (Figure 4.2). Fraction Q3 eluted at 300 mM NaCl (Figure 4.1) as a single peak. Low activity towards the synthetic substrate could be detected in fraction Q3, while in other pooled fractions no activity could be detected (data not shown). The low specific activity of fraction Q3 could be due to the delay in freeze-drying the heat-treated enzyme solution (~2 L) since it was kept for 3 days at 4°C because of freeze-drying problems.

Fraction Q3 was recovered with a yield of 16.7% and a purification factor of 0.4-fold due to the effect of tryptic inhibitors in this fraction. Fraction Q3 was resolved via

hydroxylapatite chromatography and an active fraction (Q3HA2) was eluted as a single peak at 20 mM borate-phosphate buffer (pH 7.5).

Ohkubo *et al.* (2004a) obtained a purification factor of 130-fold with a recovery of 6.3% from Q-Sepharose being a strong anion-exchanger. However, this purification attempt in the author's study was affected by a delay of the crude extract to be lyophilized before the first purification step as mentioned above. Ohkubo *et al.* (2004a) showed a purification factor of 1261-fold for lizard fish MBSP, with recovery of 6.9%, and a specific activity of 206.1 U/mg using phenyl-Sepharose, as a final step of purification, while in this study hydroxylapatite was used, which gave a specific activity of 11.2 U/mg (Table 4.1). However, the enzyme activity detected towards Boc-Phe-Ser-Arg-MCA substrate was not sufficient for further purification of ostrich MBSP. The M_r of partially purified ostrich MBSP was estimated to be 21 kDa under non-reducing conditions (result not shown) and gave 3 major bands under reducing conditions (Figure 4.3). MBSP's M_r values (native molecule), ranging from 28-120 kDa, have been reported for various fish species and mouse skeletal muscle (Cao *et al.,* 2006a; Ohkubo *et al* 2004a; Sangorrin *et al.*, 2002) (see Table 1.1). Fraction Q3HA2 could not be used for further analysis due to a loss in activity.

4.1.2 SECOND ISOLATION AND PURIFICATION ATTEMPT

4.1.2.1 Isolation procedure

Ostrich MBSP was isolated from skeletal muscle according to Sangorrin *et al.* (2002). Two kilograms of frozen muscle was thawed at 37˚C, trimmed of fat and cut into small pieces. The skeletal muscle was minced with the use of an electric meat mincer. The minced meat (1.6 kg) was mixed with 4 volumes of 20 mM Na_2PO_4/100 mM NaCl/1 mM EDTA buffer (pH 7.5) and homogenized using a Waring blender for 30 s (3x) with 10 s intervals. The homogenate was centrifuged at 500xg for 15 min. The supernatant was discarded obtaining a creamy layer (myofibrillar fraction) and the pellet was resuspended in 4 volumes of the same buffer and centrifuged as above. The resultant myofibrillar fractions were combined and the pellet was discarded. The myofibrillar fraction was washed 3 times with 4 volumes of the homogenizing buffer by gentle stirring with an overhead mechanical stirrer for 5 min and centrifuged at 500xg for 15 min.

The washed myofibrillar fraction (1 L) was suspended in 7 volumes of 50 mM Tris-HCl/100 mM KCl buffer (pH 8.5), stirred overnight with an overheard mechanical stirrer and was concentrated 4-fold with PEG 20000. Ostrich MBSP was isolated from the concentrated myofibrillar fraction (2005 ml) by adding ice cold 40% (v/v) ethylene glycol, stirring for 1 h using an overhead mechanical stirrer, followed by centrifugation at 12000xg for 20 min. The supernatant (2080 ml) was collected and adjusted to pH 4.4 with 7.5% (v/v) acetic acid to precipitate unneeded proteins and centrifuged at 12000 xg for 15 min. The supernatant (2850 ml) was adjusted to pH 8.5 with 100 mM NaOH to a final volume of 2900 ml and dialyzed overnight against 20 mM Tris-HCl buffer (pH 7.5) to remove ethylene glycol. The dialyzed enzyme solution was concentrated 4-fold against PEG 20000 overnight. The concentrated solution (690 ml) (ST) was dialyzed overnight against 20 mM Tris-HCl buffer (pH 7.5).

4.1.2.2 Column chromatography purification steps

The crude ostrich MBSP was purified by two chromatographic steps. The enzyme was partially purified by IEC using Toyopearl Super Q 650S, followed by p-aminobenzamidine-Agarose affinity chromatography.

4.1.2.2.1 Toyopearl Super Q 650S chromatography

Toyopearl Super Q 650S resin was regenerated and equilibrated as outlined in section 3.7.2.1.1. The dialyzed crude enzyme solution (690 ml) obtained in section 4.1.2.1 was applied batch-wise to 200 ml Toyopearl Super Q resin equilibrated with equilibration buffer (20 mM Tris-HCl, pH 7.5). The resin and crude enzyme were allowed to interact by slowly stirring overnight with an overhead mechanical stirrer. Unadsorbed proteins were removed by centrifugation at 1000xg for 10 min. The resin with adsorbed proteins was washed with 8 volumes of equilibration buffer by gently stirring for 2 h with a mechanical overhead stirrer, the resin was allowed to settle for 1 h and the supernatant was discarded. The latter washing step was repeated 3 times subsequently.

The extensively washed resin was packed into a glass column (2.6 x 38 cm). The column was washed overnight with approximately 2 L of the equilibration buffer at 10 ml/h and the protein content was monitored until a constant A_{280nm} value was reached. The adsorbed proteins were eluted by a linear salt gradient from 0 to 500 mM NaCl (total volume 2 L) in the same buffer at 160 ml/h. Pooled fractions were concentrated with PEG 20000, dialyzed against 20 mM Tris-HCl buffer (pH 7.5) and assayed for enzyme activity according to Ohkubo *et al.* (2004a). The fractions were stored in 50% (v/v) ethylene glycol and kept at -20˚C until used for further purification.

4.1.2.2.2 p-Aminobenzamidine-Agarose chromatography

p-Aminobenzamidine-Agarose was regenerated and equilibrated as outlined in section 3.7.5.1.3. Pooled and dialyzed fractions obtained in section 4.1.2.2.1 were applied to a p-aminobenzamidine-Agarose column (1 x 6.4 cm), equilibrated with the equilibration buffer (10 mM Tris-HCl, pH 8) at 5 ml/h. Unadsorbed proteins were eluted with the same

buffer until A_{280nm} reached a baseline value. Adsorbed proteins were eluted by the same buffer containing 0.5 M NaCl.

4.1.2.3 Results and discussion

The ethylene glycol extract was applied batch-wise to the Toyopearl Super Q 650S resin; therefore, the breakthrough peak could not be shown on the elution profile (Figure 4.4.).
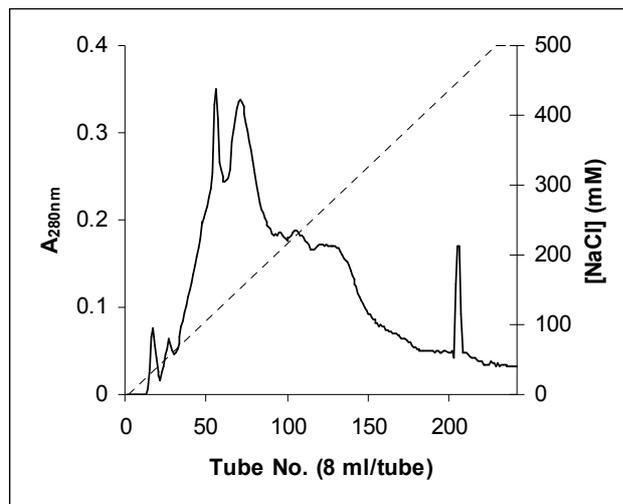
Figure 4.4. Toyopearl Super Q 650S chromatography of the ethylene glycol extract (fraction ST) (222.3 mg). Column: 2.6 x 38 cm. Linear gradient: 0-500 mM NaCl in 20 mM Tris-HCl (pH 7.5). Tubes were pooled: 17-35 (ST1); 36-66 (ST2); 67-90 (ST3); 91-123 (ST4); 124-152 (ST5); 153-216 (ST6). —A_{280nm}; --- [NaCl].

p-Aminobenzamidine-Agarose chromatography is a classical final purification step of MBSP. Figure 4.5 represents the elution profiles of fractions ST 1-6 using p-aminobenzamidine-Agarose chromatography.
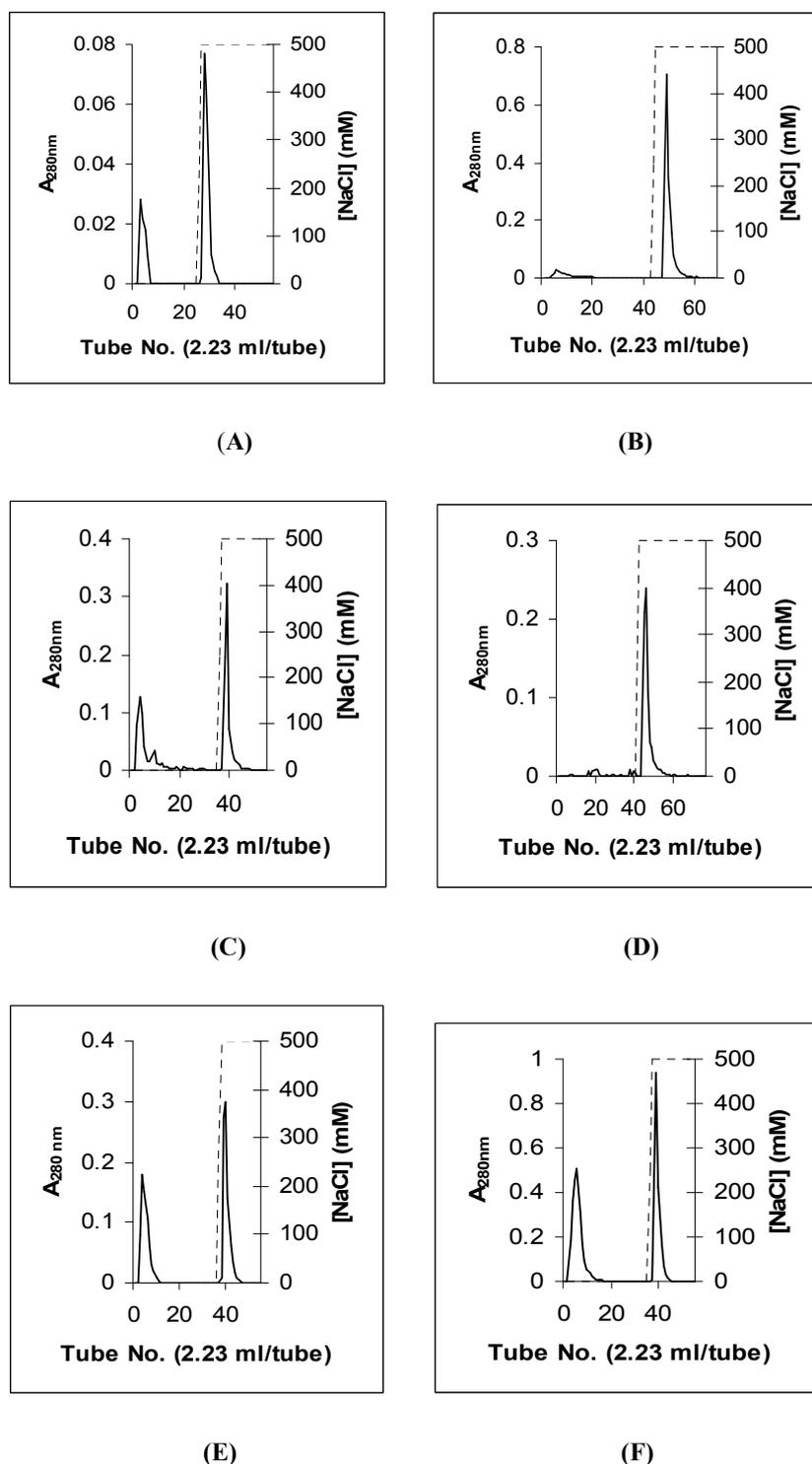
Figure 4.5. p-Aminobenzamidine-Agarose chromatography of fractions obtained from Toyopearl Super Q 650S chromatography. Column: 1 x 6.4 cm. Step-wise gradient: 0.5 M NaCl in 10 mM Tris-HCl buffer (pH 8). (A) ST1**,** (B**)** ST2, (C) ST3**,** (D) ST4, (E) ST5, (F) ST6. In A-F, tubes representing A_{280nm} peaks, immediately following introduction of 0.5 M NaCl, were pooled, e.g. ST6AB2, as well as the breakthrough peaks, e.g. ST6AB1.

Figure 4.6 represents hydrolysis of the fluorogenic synthetic substrate Boc-Phe-Ser-Arg-MCA by various concentrations of ostrich MBSP.

(A)   (B)

(C)   (D)
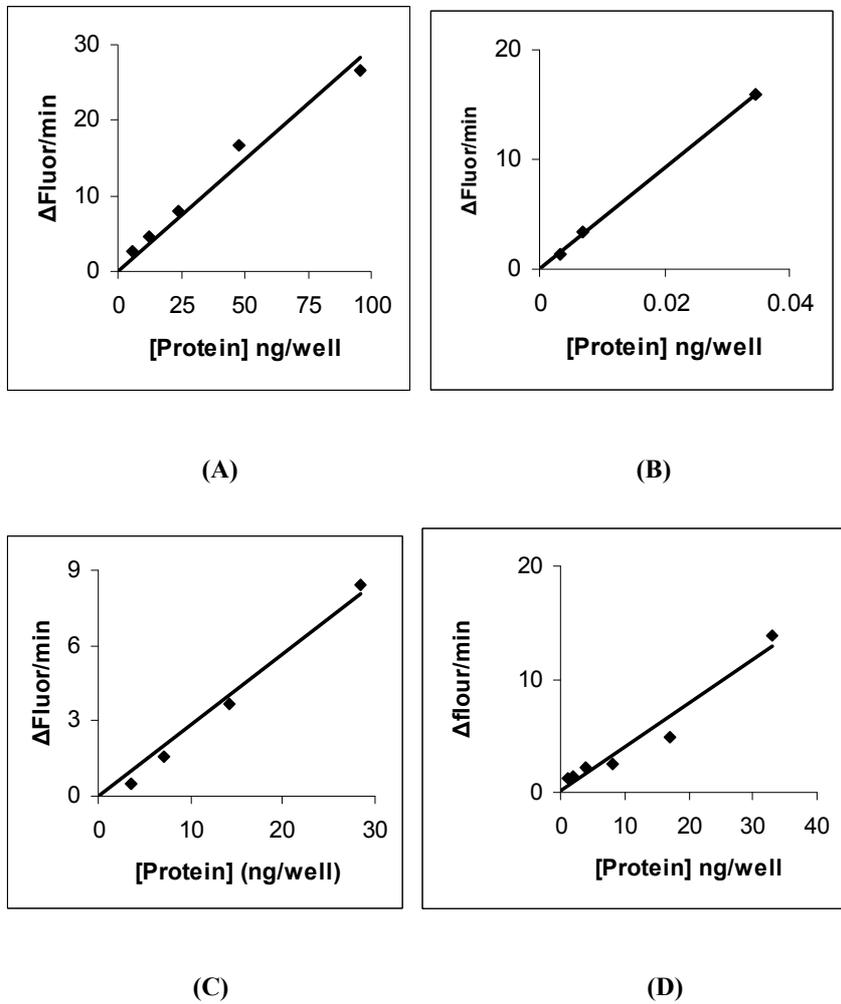
Figure 4.6. Hydrolysis of Boc-Phe-Ser-Arg-MCA versus protein concentrations of ostrich MBSP. (A) ST3AB2 (r^2=0.9857), (B) ST4AB2 (r^2=0.999), (C) ST5AB2 (r^2=0.985), (D) ST6AB2 (r^2=0.95).

A summary of second isolation and purification attempt is shown in Table 4.2

Table 4.2: Summary of the second isolation and purification procedure of ostrich MBSP from 1.6 kg minced skeletal muscle.

Purification step	Fraction code	Volume (ml)	Total Protein (mg)	Total activity (U)*	Specific activity (U/mg)*	Purifica-tion (fold)	Enzyme yield (%)	Inhibi-tion (%)
Ethylene glycol extract	ST	690	222.3	75.9	0.34	1	100	-
Toyopearl Super Q 650S	ST6	8.0	19.5	47.0	2.4	7.1	61.9	3.4
p-Aminobenz-amidine-Agarose	ST6AB2	1.05	1.266	5034	3976.3	11695	>100	-

* U= ΔFluorescence/min

After p-aminobenzamidine-Agarose chromatography of fraction ST6, a purification of 11695-fold was obtained with a recovery of more than 100% (Table 4.2), suggesting that ethylene glycol extraction dissociated MBSP with some inhibiting components that could not be well separated from the enzyme by Toyopearl Super Q chromatography. This assumption is supported by a low tryptic inhibition (3.4%), which was detected in fraction ST6 and all other fractions from Figure 4.4, revealing low enzyme activities, followed by a dramatic increase in specific activities after p-aminobenzamidine-Agarose chromatography, resulting in higher than 100% enzyme recoveries (data not shown).

SDS-PAGE patterns of fractions resulting from Toyopearl Super Q 650S chromatography (ST 1-6) are depicted in Figure 4.7.
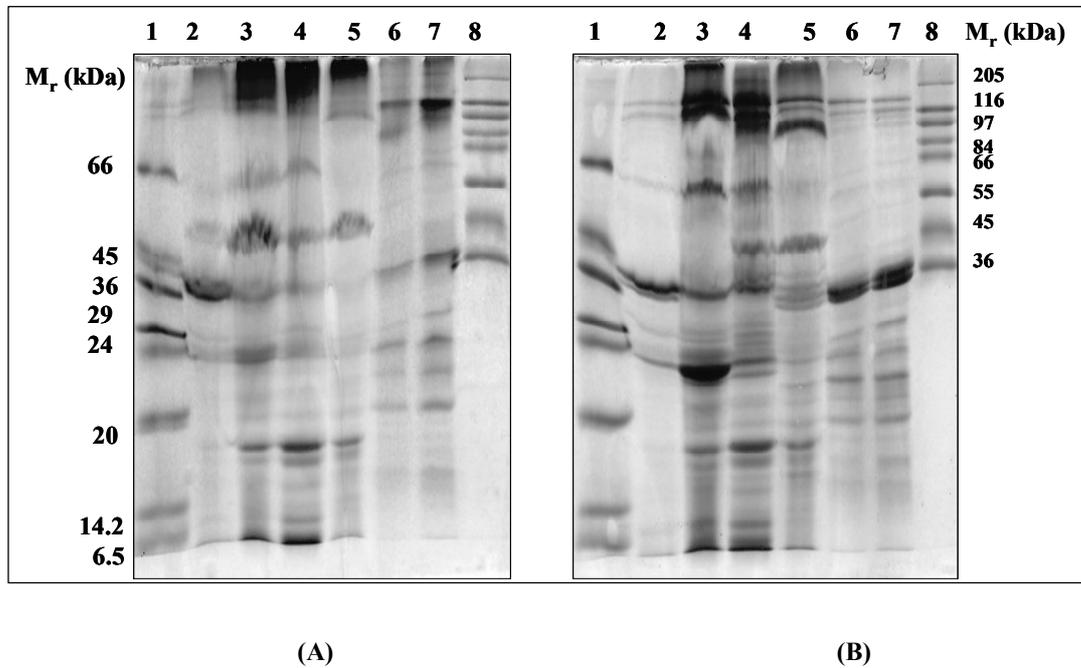
Figure 4.7. SDS-PAGE patterns of fractions resulting from Toyopearl Super Q 650S chromatography. Fractions (10 µg/lane) were loaded under (A) non-reducing and (B) reducing conditions. Lanes: 1, LMW markers; 2, ST1; 3, ST2; 4, ST3; 5, ST4; 6, ST5; 7, ST6; 8, HMW markers (see Figure 4.4).

SDS-PAGE patterns of fractions of the second isolation attempt are depicted in Figure 4.8.

(A) **(B)**
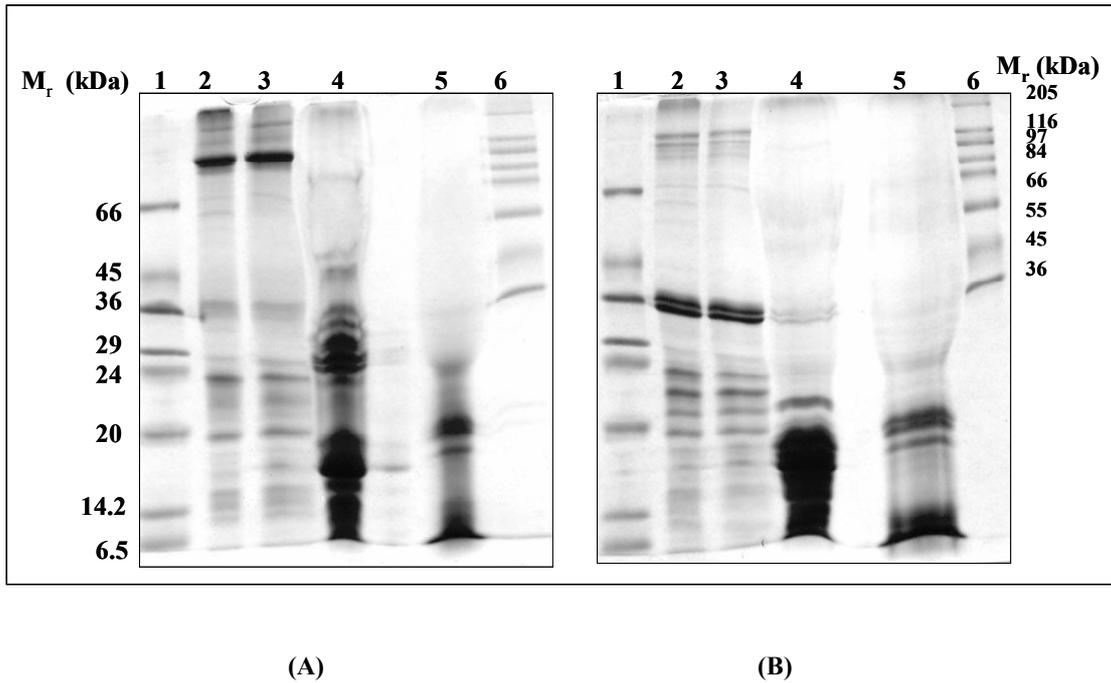
Figure 4.8. SDS-PAGE patterns of fractions resulting from p-aminobenzamidine-Agarose chromatography. Fractions (10 μg/lane) were loaded under (A) non-reducing and (B) reducing conditions. Lanes: 1, LMW markers; 2, ST6; 3, ST6 (dialyzed); 4, ST6AB1; 5, ST6AB2; 6, HMW markers (see Figure 4.5 F).

Caseinolytic zymography of fraction Q2Su3HQ6 is depicted in Figure 4.9
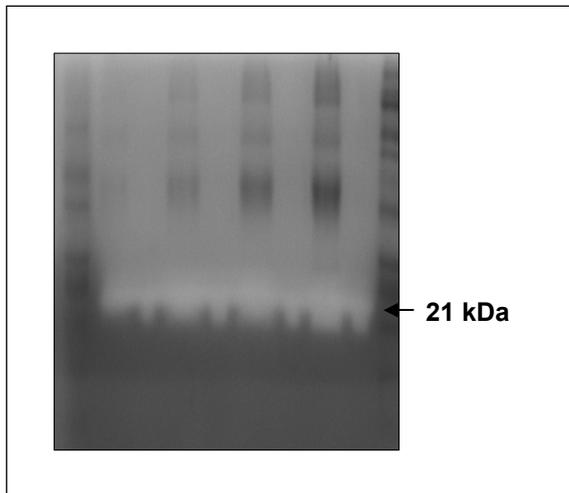
Figure 4.9. Caseinolytic zymogram of partial purified ostrich MBSP. Fraction Q2Su3HQ6 (10μg/well) was loaded under non-reducing conditions. The sample used for zymography analysis was partially purified on Toyopearl Super Q 650S and Superdex 200 HR prep grade 26/60 connected to an ÄKTA FPLC system (data not shown). Various MBSP concentrations (2.5, 5, 10 and 20 μg/lane) were loaded. Arrow indicates a proteolysis zone by ostrich MBSP.

Ostrich MBSP was isolated from washed myofibrils using 40% (v/v) ethylene glycol, followed by column chromatographies using Toyopearl Super Q 650S and p-amino-benzamidine-Agarose, the latter in place of hydroxylapatite as a final purification step. The elution of the Toyopearl Super Q 650S column was carried out with a linear gradient of NaCl (Figure 4.4). The purification results are summarized in Table 4.2. Approximately 1.27 mg of partially purified MBSP was obtained from 1.6 kg of skeletal muscle. Comparing heat- and alcohol-treated crude extract (fraction Q) (Table 4.1) with ethylene glycol extract (Table 4.2), the specific activity of the ethylene glycol extract (fraction ST) was 43% of the fraction Q value. Fractions ST and Q yielded specific activity values of 0.34 and 0.8 U/mg, respectively. Low MBSP activity towards the synthetic substrate could be detected in all pooled fractions except in fraction ST5, to which some tryptic-like inhibitors were bound (data not shown).

An increase in ostrich MBSP specific activity of 7.1-fold with a recovery of 61.9% was obtained after Toyopearl Super Q 650S chromatography (fraction ST6), in comparison with a decrease in specific activity of 0.4-fold with a recovery of 16.7% (fraction Q3). Sangorrin *et al.* (2002) on the other hand, obtained an increase in specific activity of 38-fold with a recovery of 20% for protease M using Mono Q chromatography. During p-aminobenzamidine-Agarose chromatography an active fraction (ST6AB2) was eluted as a single peak at 500 mM NaCl (Figure 4.5F), showing a purification factor of 11695-fold with a recovery of more than 100%. The very high increase in specific activity of fraction ST6AB2 is probably due to the removal of an endogenous inhibitor. Therefore, p-aminobenzamidine-Agarose chromatography proved to be a good substitute for hydroxylapatite chromatography. Sangorrin *et al.* (2002) used Superose 12 after, and not in place of hydroxylapatite chromatography, and obtained a purification of 75-fold with a recovery of 8%, a specific activity of 150.0 U/mg and 0.24 mg of purified protease M from undefined pooled muscle from fourteen mice (25 g body weight). The hydrolysis of Boc-Phe-Ser-Arg-MCA by various low protein concentrations of partially purified ostrich MBSP was evaluated (Figures 4.6A-D), yielding an highly active fraction (ST6AB2) that was used for partial characterization.

Fractions ST5 and ST6 revealed a trimer of M_r 116 kDa under non-reducing conditions and monomers with M_r values of 36 and 35 kDa under reducing conditions (Figure 4.7). Fraction ST6AB2 showed a M_r of 21 kDa (major band) under non-reducing conditions and 3 major bands with M_r values of 21, 20 and 19 kDa under reducing conditions (Figure 4.8). The M_r values for ostrich MBSP from this purification attempt agreed with the M_r detected from the first attempt. Interestingly, a high M_r component (native molecule) in the active fraction was completely disrupted after further purification on affinity chromatography and it showed several bands under non-reducing conditions, suggesting that an enzyme was tightly bound to an unknown natural substrate from myofibrils, which cannot be dissociated from the enzyme by both ethylene glycol and heat treatments.

This result strongly agrees with findings of Cao *et al*. (2006a), who detected a band of 97 kDa under non-reducing conditions and 34 kDa under reducing conditions in a partially purified enzyme from the skeletal muscle of yellow croaker (*Pseudosciaena crocea*). Furthermore, a single band of M_r 34 kDa under non-reducing conditions was detected after a further purification step on RP-HPLC using a Bio-Sil SEC-125 column in which yellow croaker MBSP purity was difficult to be detected during SDS-PAGE due to its extremely low concentration (Cao *et al*., 2006a). It was suggested that a single band of 97 kDa under non-reducing conditions is a combination of MBSP (28 kDa) and the dimer form of α-tropomyosin (68 kDa); therefore, the purified enzyme was named tropomyosin-bound serine proteinase (Cao *et al*., 2006a). The enzyme was named after N-terminal amino acid sequence analysis of the highly purified protein revealed that it shared a 90% identity to α-tropomyosin from smooth muscle of white croaker (*P. argentata*), human and rat, and western blot analysis using anticarp α-tropomyosin polyclonal antibody also showed positive results (Cao *et al*., 2006a). On the other hand, Sangorrin *et al*. (2002) reported Protease M with M_r 120 kDa after ethylene glycol extraction and partial purification by ion exchange chromatography on Mono Q and gel filtration on Superose 12, but its purity could not be clearly detected on SDS-PAGE. Guo *et al*. (2007) successfully purified MBSP from the skeletal muscle of Crucian carp

(*Carassius auratus*), which revealed an M_r of 28 kDa under reducing and non-reducing conditions.

The isolation and purification procedure of the second attempt seems to be the best of all MBSP purification procedures that have been reported, due to high specific activity and recovery, even though ethylene glycol seems to extract both natural substrate and tryptic-like inhibitor. All other pooled fractions from Toyopearl Super Q chromatography were also purified on affinity chromatography (Figure 4.5) and resulted in high ostrich MBSP specific activities towards the synthetic substrate. Furthermore, major active pooled fractions showed protein dependent enzyme activity towards the hydrolysis of the synthetic substrate (Figure 4.6). Despite the fact that the breakthrough peak showed high specific activity, the elution procedure on p-aminobenzamidine-Agarose was successful (data not shown). Ostrich MBSP obtained in this purification attempt also corresponds to a proteolysis clear band that revealed an M_r value of 21 kDa on casein SDS-PAGE zymography (Figure. 4.9). The latter study was conducted using a partially purified ostrich MBSP from skeletal muscle and due to the low protein concentration of the fraction, SDS-PAGE results without zymography could not be shown. Further attempts were undertaken to gain more knowledge of other isolation methods and chromatographic steps for the purification of ostrich MBSP to homogeneity.

4.1.3 THIRD ISOLATION AND PURIFICATION ATTEMPT

4.1.3.1 Isolation procedure

Ostrich MBSP was isolated from skeletal muscle according to Osatomi *et al.* (1997). All the steps were carried out at 4°C except where mentioned otherwise. Frozen ostrich skeletal muscle (845 g) was thawed at 37°C, trimmed of fat and minced with the use of a meat mincer. The minced meat (0.5 kg) was suspended in 4 volumes of 25 mM potassium-phosphate buffer (pH 7.5) and gently stirred for 2 min with the use of an overhead mechanical stirrer. The suspension was centrifuged at 8000xg for 10 min to remove soluble matter. The supernatant was discarded and the insoluble matter was washed four times with the same buffer by centrifugation under the same conditions. The washed precipitate was suspended in 4 volumes of 25 mM potassium-phosphate buffer (pH 6.4) containing 500 mM KCl, 1 mM $MgCl_2$ and 5 mM $Na_4P_2O_7$, and homogenized at a slow speed using a Waring blender for 2 x 30 s with 10 s intervals. The homogenate (1.95 L) was filtered through cheese cloth to remove fibrous matter after standing for 30 min.

The filtrate was divided into five equal volumes and each volume was suspended in 15 volumes of ice cold distilled water and allowed to stand overnight. The resulting precipitate (myofibril fraction) was collected by centrifugation at 10000xg for 30 min, while the supernatant was discarded. The myofibril fraction (1 L) was suspended in 3 volumes of 2 M KCl and adjusted to pH 4.0 with 3 M HCl by gently stirring with a glass rod. The myofibril suspension was allowed to stand for 2 h and thereafter, the solution was centrifuged at 8000xg for 10 min. The supernatant (3.25 L) was collected and the precipitate (200 ml) was resuspeded in 5 volumes of 2 M KCl and centrifuged. Both supernatants were combined (5.25 L) as a crude MBSP fraction and concentrated 6-fold for 16 h with PEG 20000. The concentrate was dialyzed overnight against ice cold dH_2O water. The dH_2O was changed 3 times during the dialysis process to remove salts completely and the enzyme solution (860 ml) was lyophilized. The crude enzyme was dissolved in 25 ml 5 mM sodium acetate buffer (pH 4.0) containing 400 mM NaCl and centrifuged at 10000xg for 10 min. The supernatant (crude MBSP) (SG fraction) was dialyzed overnight against the same buffer to remove excess KCl.

4.1.3.2 Column chromatography purification steps

Crude ostrich MBSP was purified by three chromatographic steps, namely gel exclusion chromatography on Sephadex G-75, followed by affinity chromatography on arginine-Sepharose CL-4B and hydrophobic interaction chromatography on phenyl-Sepharose CL-6B fast flow.

4.1.3.2.1 Sephadex G-75 chromatography

Sephadex G 75 resin was prepared and equilibrated as mentioned in section 3.7.3.1.1. The dialyzed crude MBSP (149 mg/25 ml), obtained in section 4.1.3.1, was applied to a Sephadex G-75 column (2.6 x 95 cm) equilibrated with 5 mM sodium acetate buffer (pH 4.0) containing 400 mM NaCl at 10 ml/h. Protein was eluted with the same buffer at 12 ml/h. Pooled fractions were concentrated with PEG 20000, dialyzed against 10 mM Tris-HCl buffer (pH 7.5) and assayed for MBSP activity.

4.1.3.2.2 L-Arginine-Sepharose chromatography

L-Arginine-Sepharose CL-4B resin was prepared and equilibrated as illustrated in section 3.7.5.1.1. Fraction SG2 obtained in section 4.1.3.2.1, was purified further on arginine-Sepharose CL-4B equilibrated with 10 mM Tris-HCl buffer, pH 7.5. Fraction SG2 was applied to a column of L-arginine-Sepharose (1 x 8 cm) at 5 ml/h. Unadsorbed proteins were eluted with the same buffer, while the adsorbed ones were eluted by a step-wise gradient elution (0.2, 0.5 and 1 M KCl). Pooled fractions were concentrated with PEG 20000, dialyzed against 10 mM Tris-HCl buffer (pH 7.5) and assayed for MBSP activity.

4.1.3.2.3 Phenyl-Sepharose chromatography

Phenyl-Sepharose CL-6B fast flow was regenerated as mentioned in section 3.7.4.1.1. The dialyzed fraction SG2Arg4 (1mg) obtained from section 4.1.3.2.2 was applied to a phenyl-Sepharose CL-6B fast flow column (1 x 2 cm) equilibrated with 10 mM borate-phosphate buffer ($Na_2B_4O_7$-KH_2PO_4) (pH 7.5), containing 1 M $(NH_4)_2SO_4$ at 5 ml/h. Unadsorbed proteins were eluted with the same buffer. Adsorbed proteins were eluted by

10 mM borate-phosphate buffer (pH 7.5) and the enzyme was eluted with 50% (v/v) ethylene glycol in the latter buffer. Pooled fractions were concentrated with PEG 20000, dialyzed against 10 mM borate-phosphate buffer (pH 7.5) and assayed for MBSP activity.

4.1.3.3 Results and discussion

Figure 4.10 represents the elution profile of a salt and acid treated extract (fraction SG) using Sephadex G-75 chromatography.
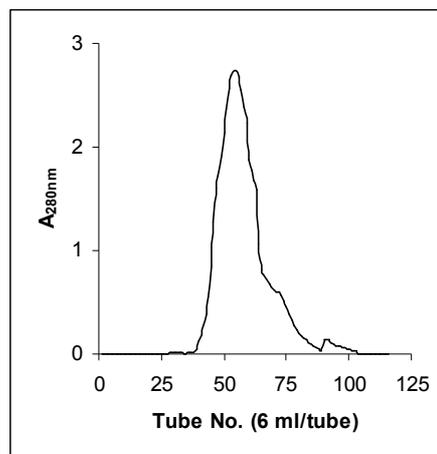
Figure 4.10. Sephadex G-75 chromatography of fraction SG (149 mg). Column: 2.6 x 95 cm. Tubes were pooled: 39-47 (SG1); 48-59 (SG2); 60-67 (SG3); 68-87 (SG4); and 90-100 (SG5). — A_{280nm}.

L-Arginine-Sepharose CL-4B affinity chromatography is a classical final purification step of MBSP. The column was tested for its affinity to bind commercial bovine trypsin, followed by purification of active ostrich MBSP (fraction SG2). However, ostrich MBSP interacted with L-arginine-Sepharose resin with some contaminants as shown in SDS-PAGE patterns (Figure 4.14). Therefore, ostrich MBSP was further purified using phenyl-Sepharose chromatography.

The elution profiles of L-arginine-Sepharose and phenyl-Sepharose chromatographies are shown in Figures 4.11 and 4.12, respectively.

(A)   **(B)**
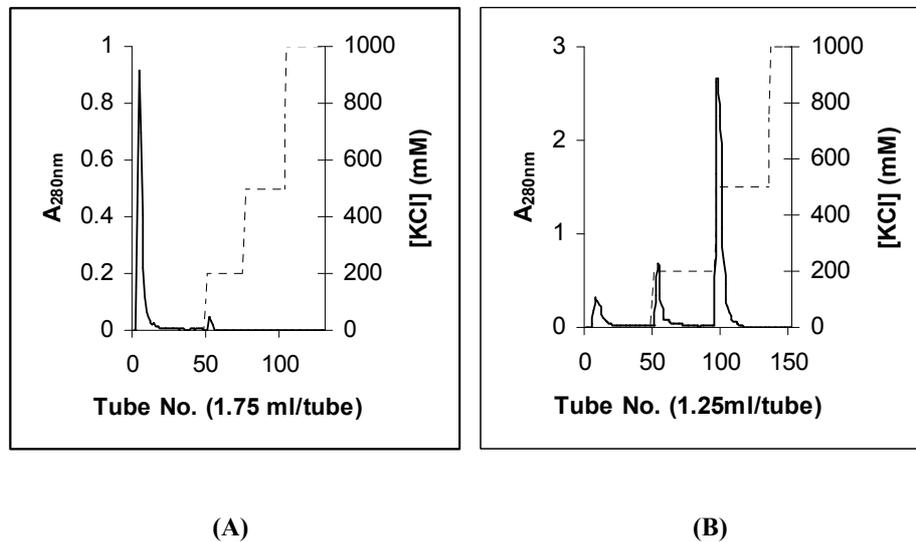
Figure 4.11. L-Arginine-Sepharose chromatography of (A) commercial bovine trypsin (5 mg) and (B) fraction SG2 (15.3 mg). Column: 1 x 8 cm. Step-wise salt gradient: 0.2, 0.5 and 1 M KCl in 10 mM Tris-HCl buffer (pH 7.5). Tubes were pooled (B): 6-9 (SG2Arg1); 53-59 (SG2Arg2); 60-72 (SG2Arg3); 73-84 (SG2Arg4); 97-105 (SG2Arg5) and 106-117 (SG2Arg6). $-$ A_{280nm}; --- [KCl].
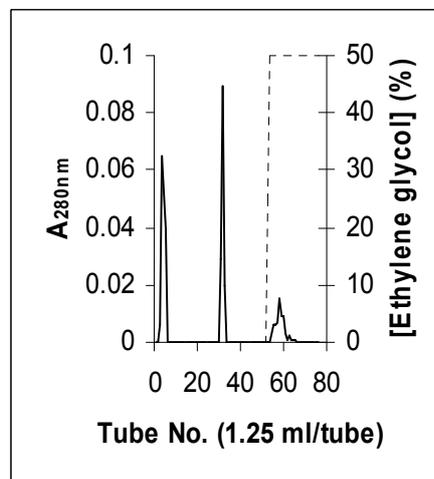
Figure 4.12. Phenyl-Sepharose chromatography of fraction SG2Arg6 (1mg). Tubes 1-25 were eluted with 10 mM borate-phosphate buffer (pH 7.5) containing 1 M $(NH_4)_2SO_4$; tubes 26-51 were eluted with borate-phosphate buffer (pH 7.5) and tubes 52-80 were eluted with 50% (v/v) ethylene glycol. Tubes were pooled: 3-4 (P1); 31-33 (P2) and 55-61 (P3). $-A_{280nm}$; --- [ethylene glycol].

A summary of the third isolation and purification attempt is shown in Table 4.3.

Table 4.3: Summary of the third isolation and purification procedure of ostrich MBSP from 500 g minced skeletal muscle.

Purification step	Fraction code	Volume (ml)	Total Protein (mg)	Total activity (U)*	Specific activity (U/mg)*	Purification (Fold)	Yield (%)
2M KCl, pH4 crude extract	SG	24	149.0	2268	15.22	1	100
Sephadex G-75	SG2	3.6	15.3	0	0	0	0
L-Arginine-Sepharose	SG2Arg2	2	0.124	184.3	1486.0	97.8	8.1

* U= ΔFluorescence/min

SDS-PAGE patterns of fractions from Sephadex G-75 chromatography (SG1-4) are depicted in Figure 4.13

(A)   (B)
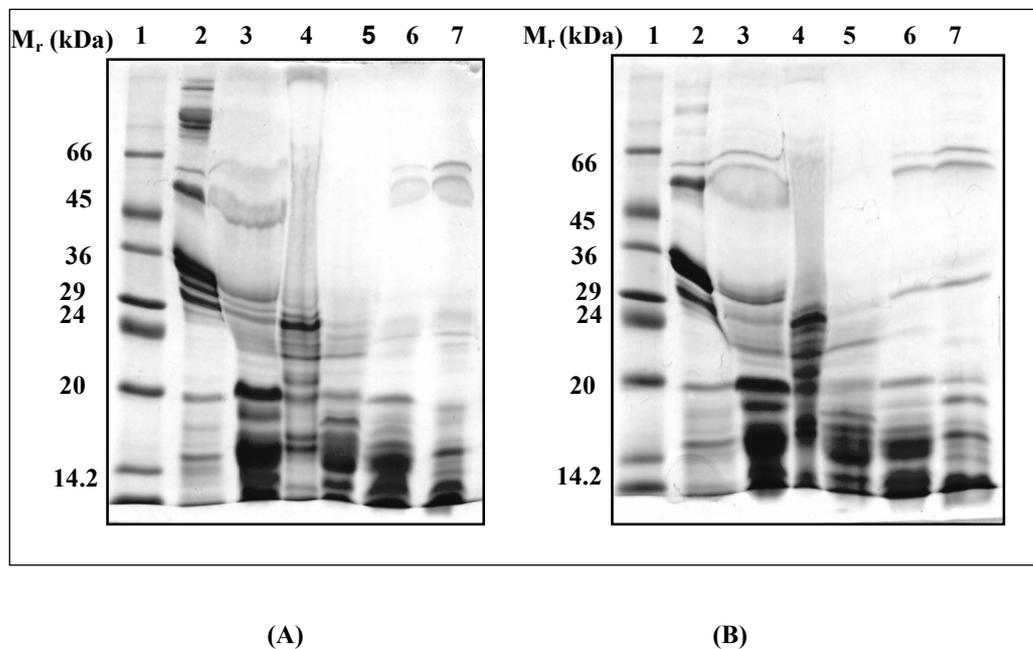
Figure 4.13. SDS-PAGE patterns of fractions resulting from Sephadex G-75 chromatography. Fractions (10 µg/lane) were loaded under (A) non-reducing and (B) reducing conditions. Lanes: 1, LMW markers; 2, SG (precipitate); 3, SG; 4, SG1; 5, SG2; 6, SG3; 7, SG4 (see Figure. 4.10).

SDS-PAGE patterns of fractions resulting from the third isolation and purification attempt are depicted in Figure 4.14.
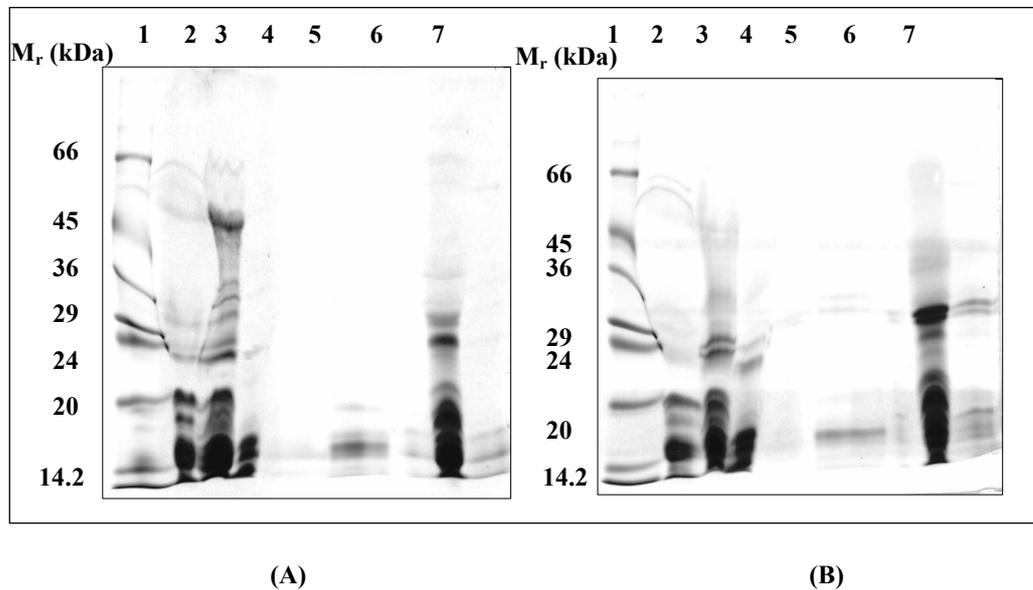
Figure 4.14. SDS-PAGE patterns of fractions resulting from third isolation and purification attempt. Fractions (10 µg/lane) were loaded under (A) non-reducing and (B) reducing conditions. Lanes: 1, LMW markers; 2, SG; 3, SG2; 4, SG2Arg6; 5, SG2Arg6P1; 6, SG2Arg6P2; (7) SG2Arg6P3 (see Figures 4.11 and 4.12).

Ostrich MBSP was isolated from washed myofibrils with 2 M KCl at pH 4, followed by two column chromatographies, Sephadex G-75 and L-arginine-Sepharose, with phenyl-Sepharose being added as an additional step (purification results not shown), since purification could not be shown during SDS-PAGE. Gel filtration was used as a first purification step in place of Toyopearl-Super Q 650S used in the first and second attempts. L-Arginine-Sepharose was used in place of p-aminobenzamidine-Agarose chromatography in the second attempt and replaced hydroxylapatite in the first attempt.

Approximately 0.124 mg of partially purified MBSP (fraction SG2Arg2) was obtained from 500 g skeletal muscle and a purification factor of 98-fold and a recovery of 8.1% (Table 4.3). The Sephadex G-75 elution profile shows that MBSP was not well separated from contaminating proteins as a huge broad peak eluted (Figure 4.10) and SDS-PAGE patterns of pooled fractions are shown in Figure 4.13. According to results obtained by Osatomi *et al.* (1997), carp MBSP could be separated from contaminating proteins by

Ultrogel AcA 54 SEC, resulting in a purification factor of 9340-fold with a recovery of 376% and specific activity of 7.73 U/mg. After Sephadex G-75 chromatography, ostrich MBSP activity could not be detected from all pooled fractions, due to the effect of a tryptic-like inhibitor in the fractions (data not shown). Amongst all pooled fractions, SG2 showed a strong inhibition (100%) toward commercial bovine trypsin (data not shown). This fraction was applied to an L-arginine-Sepharose column, which was first tested with commercial bovine trypsin (Figure 4.11A). The MBSP active fraction (SG2Arg2) was eluted at 0.2 M KCl (Figure 4.11B). During SDS-PAGE several bands were detected, indicating that arginine-Sepharose chromatography could not successfully purify ostrich MBSP. Although activity could be detected, the purity of MBSP was not satisfactory (Figure 4.14).

Ostrich MBSP was found to bind strongly to an arginine-Sepharose column with some competing protein contaminants. A peak that eluted at 0.5 M KCl was pooled separately as an ascending (fraction SG2Arg5) and descending leg (fraction SG2Arg6) and the latter fraction was applied to a phenyl-Sepharose column (Figure 4.12). Interestingly, fraction SG2Arg6P2 that eluted with borate-phosphate buffer showed 63% tryptic-like inhibition (data not shown), while ostrich MBSP activity found in fraction SG2Arg6P3, eluted with 50% (v/v) ethylene glycol, indicating that L-Arginine-Sepharose chromatography could not separate MBSP from the inhibitor. However, phenyl-Sepharose successfully separated MBSP from the inhibitor. Unfortunately, MBSP could not be detected during SDS-PAGE due to its low concentration (data not shown), suggesting that a tightly bound fraction SG2Arg6 pooled from arginine-Sepharose chromatography co-eluted with a tryptic-like inhibitor. L-Arginine-Sepharose chromatography was found to be a poor substitute for p-aminobenzamidine-Agarose chromatography, which yielded high ostrich MBSP activity in the second attempt. Sephadex G-75 was not a good substitute for Toyopearl Super Q used in this study and for Ultrogel AcA 54 SEC used by Osatomi *et al.* (1997).

The SDS-PAGE pattern of the inhibitor fraction obtained from phenyl-Sepharose chromatography (fraction SG2Arg6P3) showed M_r (29 kDa) as carp fish MBSP, reported by Osatomi *et al.* (1997). A purification factor of 3.94×10^6-fold with a recovery of 77%

and a specific activity of 3260 U/mg were obtained after purification of carp MBSP using L-arginine-Sepharose chromatography, revealing a single band of M_r 30 kDa (Osatomi *et al*., 1997). Ostrich skeletal muscle contains a rich source of MBSP inhibitors that could not be separated from its target enzyme using heat, high salt and alcohol treatments. Due to a low enzyme yield obtained from this attempt, a fourth attempt at purifying MBSP was carried out.

4.1.4 FOURTH ISOLATION AND PURIFICATION ATTEMPT

4.1.4.1 Isolation procedure

Ostrich MBSP was isolated from skeletal muscle according to Osatomi *et al.* (1997) and Ohkubo *et al.* (2004a). Fresh ostrich skeletal muscle (2.95 kg) was kept in a cold room overnight prior to the enzyme isolation procedure. The skeletal muscle was trimmed of fat and minced with the use of a meat mincer. The minced meat (1.83 kg) was suspended in 4 volumes of 25 mM potassium-phosphate buffer (pH 7.5), gently stirred for 2 min with the use of an overhead mechanical stirrer and centrifuged at 8000xg for 10 min to remove soluble matter. The supernatant was discarded and the insoluble material was washed 4 times with the same buffer. The washed precipitate was suspended in 4 volumes of 25 mM K_2PO_4 buffer (pH 6.4) containing 500 mM KCl, 1 mM $MgCl_2$ and 5 mM $Na_4P_2O_7$ and homogenized at a slow speed using a Waring blender for 2 x 30 s with a 10 s intervals. The homogenate (8 L) was filtered through cheese cloth to remove fibrous matter after standing for 30 min. The filtrate was divided into six equal volumes and each volume was suspended in 3 volumes of ice cold dH_2O and left to stand overnight.

The resulting precipitate (myofibril fraction) was collected by centrifugation at 10000 xg for 30 min and the supernatant was discarded. The myofibril fraction (3.5L) was divided into three equal volumes and each was suspended in 4 volumes ice cold dH_2O and the pH was adjusted to 6 with 1 M HCl. The suspension was heated in a 100°C water bath with gentle stirring with an overhead mechanical stirrer for 1 h until the solution reached 55°C. Thereafter, the solutions were incubated at 55°C in a water bath for 10 min with gentle stirring with mechanical overhead stirrers to dissociate MBSP from myofibrils. The heat extracts were cooled immediately in ice baths until 4°C was reached and allowed to stand overnight in the cold room. Crude MBSP was collected from the heat extracts by centrifugation at 10000xg for 20 min. The supernatant (crude MBSP) (16 L) was collected and the precipitate was discarded. The crude enzyme was concentrated 5.3-fold with PEG 20000. The concentrate (3 L) was adjusted to pH 7 with 1 M NaOH and lyophilized for 72 hrs. The lyophilized crude MBSP was dissolved in 1.5 L 1 M KCl by

gently stirring for 2 h with a glass rod. The pH was adjusted to 5 with 1 M HCl and the solution allowed to stand overnight. The precipitates were discarded after centrifugation at 10000xg for 20 min and the supernatant (1.39 L) was dialyzed overnight against basal buffer, 10 mM borate-phosphate buffer (pH 7.5), to remove KCl from the crude enzyme solution. The dialyzed crude enzyme solution was concentrated 2-fold with PEG 20000 overnight and dialyzed against the same buffer for 3 h. The dialyzed enzyme solution (650 ml) was treated with absolute ethanol (-30°C) at a ratio of 7:3 (v/v) and placed in a -10°C ice/salt bath by gently stirring with a glass rod for approximately 30 min. The mixture was centrifuged at 10000xg for 20 min at -10°C "to remove transparent jelly, which are in a transparent liquid state at room temperature" (Ohkubo *et al.,* 2004a). The supernatant (780 ml) (alcohol treated crude MBSP) was dialyzed for 7 h and concentrated 6-fold with PEG 20000 overnight. The concentrated enzyme (STQ) was dialyzed overnight against 10 mM borate-phosphate buffer (pH 7.5).

4.1.4.2 Column chromatography purification steps

Crude ostrich MBSP was purified by three chromatographic steps, namely Toyopearl Super Q 650S, phenyl-Sepharose CL-6B fast flow and p-aminobenzamidine-Agarose.

4.1.4.2.1 Toyopearl Super Q 650S chromatography

The dialyzed crude enzyme solution (2.2 g) (135 ml) obtained in section 4.1.4.1 was applied batch-wise to Toyopearl Super Q resin (200 ml), equilibrated with equilibration buffer (10 mM $Na_2B_4O_7$-KH_2PO_4, pH 7.5). A batch-wise application and column packing were performed as described in section 4.1.1.2.1. The adsorbed proteins were eluted by a linear salt gradient from 0 to 500 mM (total volume 750 ml) in the equilibration buffer at 160 ml/h, followed by step-wise elution at 1 M NaCl. Pooled fractions were pooled, concentrated with PEG 20000 and dialyzed against 10 mM $Na_2B_4O_7$-KH_2PO_4 buffer (pH 7.5) (basal buffer). Pooled fractions were assayed for MBSP activity.

4.1.4.2.2 Phenyl-Sepharose chromatography

Fractions STQT2 and STQT9 obtained from section 4.1.4.2.1 were applied separately to a phenyl-Sepharose CL-6B column (1 x 2 cm) equilibrated with 10 mM $Na_2B_4O_7$-KH_2PO_4 buffer (pH 7.5) containing 1 M $(NH_4)_2SO_4$ at 5 ml/h. Unadsorbed proteins were eluted with the same buffer, while adsorbed proteins were eluted by 10 mM $Na_2B_4O_7$-KH_2PO_4 buffer (pH 7.5), and the enzyme was eluted with 50% (v/v) ethylene glycol in the same buffer. Pooled fractions were concentrated with PEG 20000, dialyzed against 10 mM $Na_2B_4O_7$-KH_2PO_4 buffer (pH 7.5) and assayed for MBSP and inhibitory activity.

4.1.4.2.3 p-Aminobenzamidine-Agarose chromatography

Fraction STQT2P1 (0.8 mg) obtained from section 4.2.4.2.2 was applied to a p-aminobenzamidine-Agarose (1 x 6.4 cm) column equilibrated with 50 mM Tris-HCl buffer (pH 8) containing 0.5 M NaCl at 5 ml/h. Unadsorbed proteins were eluted with the same buffer until A_{280nm} reached a baseline value. Bound proteins were eluted by 10 mM HCl into tubes containing 0.1 ml 1 M Tris-HCl buffer (pH 8.5).

4.1.4.3 Results and discussion

The gradient elution profile of Toyopearl Super Q 650S chromatography is shown in Figure 4.15.
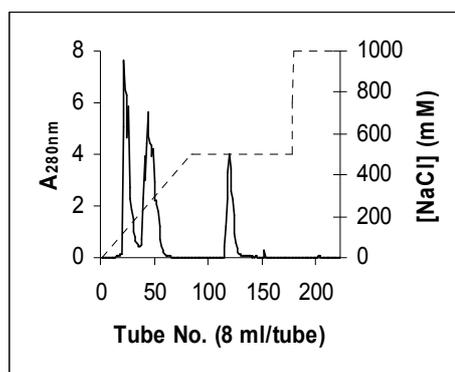
Figure 4.15. Toyopearl Super Q 650S chromatography of fraction STQ (2.2 g). Linear salt gradient: 0-500 mM NaCl in borate-phosphate buffer (pH 7.5). Tubes were pooled: 21-26 (STQT1); 27-39 (STQT2); 40-45 (STQT3); 46-53 (STQT4); 54-69 (STQT5); 70-89 (STQT6); 116-133 (STQT7); 135-153 (STQT8); 154-163 (STQT9). $-A_{280nm}$; --- [NaCl]

The elution profile of Phenyl-Sepharose chromatography of fraction STQT2 is shown in Figure 4.16.
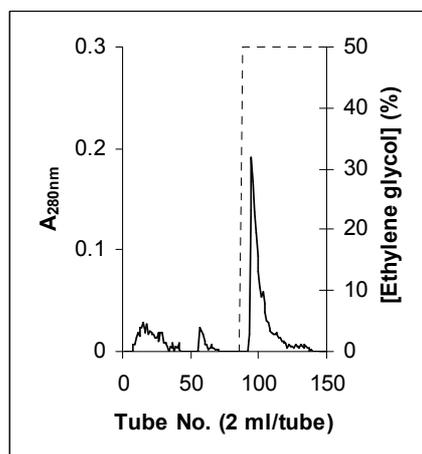
Figure 4.16. Phenyl-Sepharose CL- 6B fast flow chromatography of fraction STQT2 (5 mg). Column: 1 x 2 cm. Tubes 1-54 were eluted with 10 mM borate-phosphate buffer containing 1 M $(NH_4)_2SO_4$; tubes 55-86 were eluted with borate buffer; and tubes 87-150 were eluted by 50% (v/v) ethylene glycol. Tubes were pooled: 10-29 (STQT2P1); 57-67 (STQT2P2), 93-116 (STQT2P3); and 117-140 (STQT2P4). $-A_{280nm}$; --- [ethylene glycol]

The elution profiles of p-Aminobenzamidine-Agarose chromatography of commercial bovine trypsin and ostrich MBSP are shown in Figure 4.17.

(A)   (B)
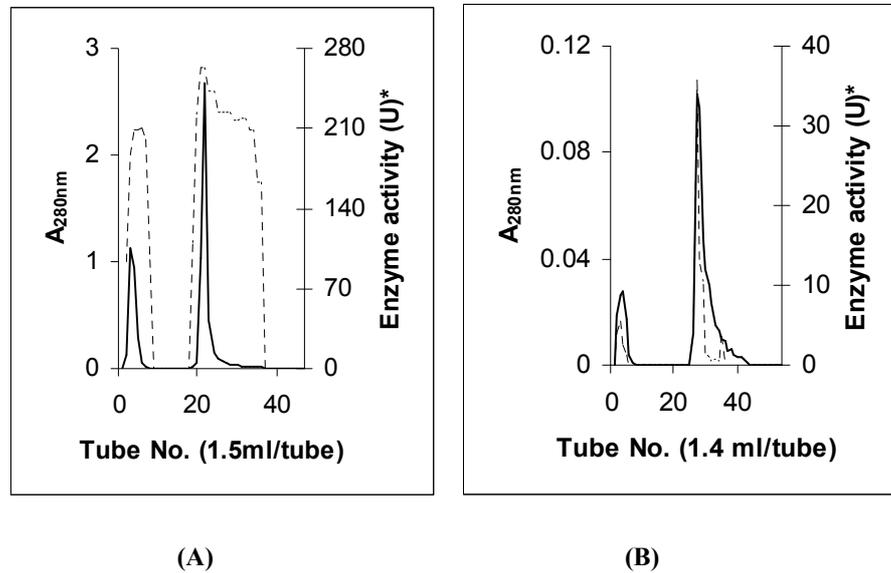
Figure 4.17. p-Aminobenzamidine-Agarose chromatography of (A) commercial bovine trypsin and (B) fraction STQT2P1 (1.3 mg). Column: 1 x 6.4 cm. Tubes 1-22 were eluted with 50 mM Tris-HCl (pH 8) and tubes 23-54 were eluted with 10 mM HCl into tubes containing 0.1 ml 1 M Tris-HCl (pH 8.5). Tubes were pooled (B): 1-5 (STQT2P1A1); 26-30 (STQT2P1A2); 31-34 (STQT2P1A3). ─A_{280nm}; --- U* = ΔFluorescence/min.

Summary of the fourth isolation and purification procedure of ostrich MBSP is shown in Table 4.4.

Table 4.4: Summary of the fourth isolation and purification procedure of ostrich MBSP from 1.83 kg minced skeletal muscle.

Purification step	Fraction code	Volume (ml)	Total Protein (mg)	Total activity (U)*	Specific activity (U/mg)	Purification (Fold)	Yield (%)
Crude extract	STQ	135	2200	1.4	0.001	1	100
Toyopearl Super Q	STQT2	3.7	168.1	56.5	0.34	340	>100
Phenyl-Sepharose	STQT2P1	1.2	1.88	7.9	4.2	4200	>100
p-Aminobenz-amidine-agarose	STQT2P1A2 STQT2P1A3	0.1 0.13	0.019 0.016	424.9 43.6	22363.0 2725.0	22.4×10^6 2.7×10^6	>100 >100

*U=ΔFluorescence/min

SDS-PAGE patterns of fractions resulting from Toyopearl Super Q 650S chromatography of the fourth isolation attempt are depicted in Figure 4.18.
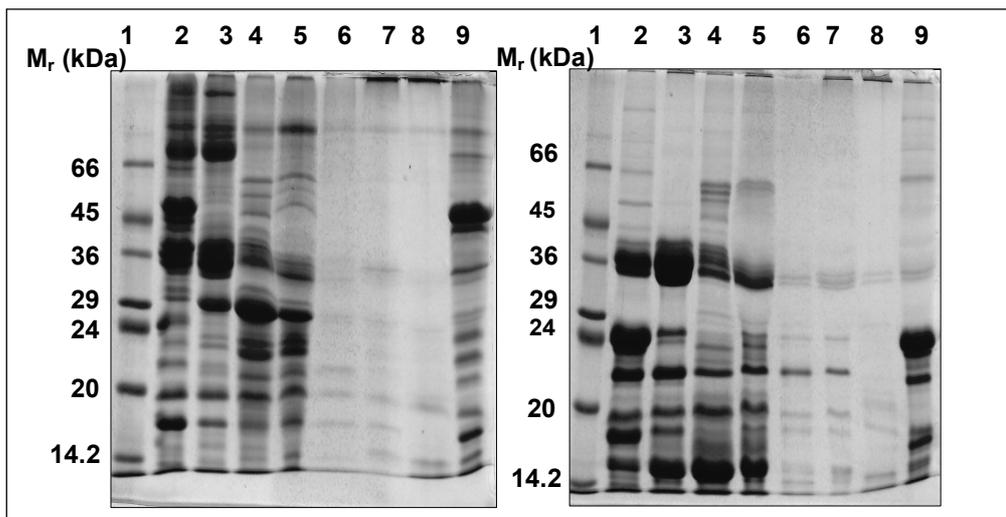
(A)   (B)

Figure 4.18. SDS-PAGE patterns of fractions resulting from Toyopearl Super Q chromatography. Fractions (10 μg/lane) were loaded under (A) non-reducing and (B) reducing conditions Lanes: 1, LMW markers; 2, STQT1; 3, STQT2; 4, STQT3; 5, STQT5; 6, STQT6; 7, STQT7; 8, STQT8; 9, STQT9.

SDS-PAGE patterns of fractions resulting from phenyl-Sepharose chromatography of the fourth isolation attempt are depicted in Figure 4.19.
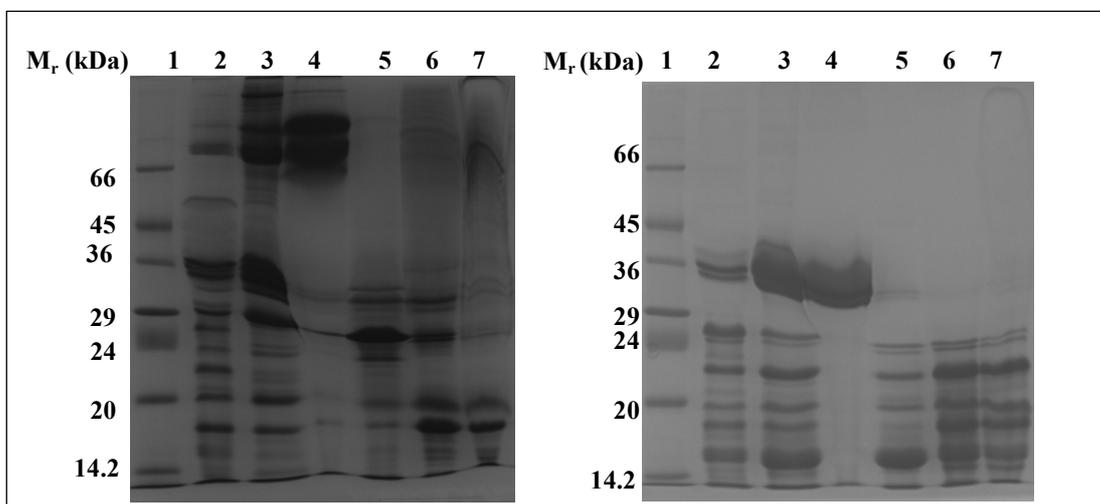
(A)   (B)

Figure 4.19. SDS-PAGE patterns of fractions resulting from phenyl-Sepharose chromatography. Fractions (10 μg/lane) were loaded under (A) non-reducing and (B) reducing conditions Lanes: 1, LMW markers; 2, STQ; 3, STQT2; 4, STQT2P1; 5, STQT2P2; 6, STQT2P3; 7, STQT2P4

The purpose of the fourth isolation and purification procedure was to improve the purification and yield of ostrich MBSP. Previous procedures employed ethylene glycol and high salt for dissociation of the enzyme from myofibrils, respectively. Toyopearl-Super Q, phenyl-Sepharose and p-aminobenzamidine-Agarose were used to further purify crude MBSP. The isolation and purification results of ostrich MBSP are summarized in Table 4.4. This purification procedure was not productive compared to the previous isolation attempts. Fraction STQT2 showed a 340-fold purification with a recovery of more than 100%. High specific activity was influenced by the removal of inhibitor and other contaminants by p-aminobenzamidine-Agarose that were found to be more dominant after dissociating MBSP from myofibrils with the combination of salt and heat treatments.

Fraction STQT2, resulting from Toyopearl Super Q chromatography (Figure 4.15), was applied to a column of phenyl-Sepharose. MBSP activity was eluted as a breakthrough peak in the presence of 1 M $(NH_4)_2SO_4$ (Figure. 4.16). MBSP (fraction STQT2P1) was further purified via p-aminobenzamidine-Agarose chromatography. High ostrich MBSP activity was resolved as a single peak, divided into two fractions, STQT2P1A2 and STQT2PA3 (Figure 4.17B). As a result, MBSP successfully interacted with the affinity column as compared to the elution of commercial bovine trypsin, which was also resolved as a single peak (Fig 4.17A). Enzyme seems to be eluted in the breakthrough peak as enzyme activity was also detected, suggesting that the column was overloaded (Figure 4.17A). However, the combination of ethylene glycol and high salt isolation procedures showed that they dissociate enzyme from myofibrils with high M_r components. Those components were tightly bound to the target enzyme, were co-eluted in the breakthrough peak of phenyl-Sepharose column, and were detected as two major bands on SDS-PAGE (Figure 4.19). The results could not be compared to other purification attempts as the specific activity was improved by extremely low protein yield, which was 35 µg in total protein (Table 4.19). The purified enzyme could not be analyzed on SDS-PAGE.

The results of fourth isolation and purification attempt strongly support the suggestion of Cao *et al.* (2007) that the binding pattern of MBSP and target binding proteins in myofibrils has been long of interest and not much evidence is available.

4.2 ISOLATION AND PURIFICATION OF ENDOGENOUS OSTRICH MBSP INHIBITOR

4.2.1 Isolation procedure

The first supernatant obtained from the fourth attempt of the isolation of ostrich MBSP (section 4.1.4.1) was used for the isolation and purification of ostrich MBSPI using the method of Cao *et al.* (2001). The MBSPI was fractionated from the sarcoplasmic fraction by 75% ammonium sulfate (AS) saturation and allowed to stand overnight. The resulting precipitate was collected by centrifugation at 12000xg for 30 min. The precipitate was dissolved in 20 mM borate buffer (pH 6) and dialyzed overnight. The dialyzed inhibitor solution was lyophilized and kept at 4°C until used.

4.2.2 Column chromatography purification steps

Three chromatographic steps were used to purify the dialyzed crude ostrich MBSPI, namely Toyopearl Super Q 650S, Superdex 200 and HiTrap SP HR chromatography

4.2.2.1 Toyopearl Super Q 650S chromatography

The crude MBSPI (fraction AS) was added to Toyopearl Super Q 650S resin (200 ml), equilibrated with 10 mM Tris-HCl buffer (pH 7.5). The sample was mixed with the equilibrated resin and slowly stirred overnight. The supernatant was collected by centrifugation at 500xg for 10 min and lyophilized. The lyophilized fraction (AST1) was dissolved in the equilibration buffer for SEC and divided into two volumes (fractions AST1c and AST1d).

4.2.2.2 Superdex 200 HR chromatography

A Superdex 200 column (2.6 x 60 cm) was connected to an ÄKTA FPLC system and equilibrated by two bed volumes of 20 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl. Fractions AST1c and AST1d were filtered using 0.22 μm syringe filters and loaded separately at 12 ml/h. The column was run at l ml/min and 2 ml fractions were collected. Adsorbed proteins were eluted with the same buffer.

4.2.2.3 HiTrap SP HR chromatography

Active MBSPI fractions AST1cS2 and AST1dS4, pooled from SEC, were dialyzed against equilibration buffer (50 mM ammonium acetate, pH 5), filtered using 0.22 µm syringe filters and loaded separately onto one 5 ml HiTrap SP columns (1.6 x 2.6cm) connected to an ÄKTA FPLC system, equilibrated with the same buffer at 12 ml/h. The column was run at l ml/min and 2 ml fractions were collected. Unadsorbed proteins were eluted with the same buffer and bound proteins were eluted with a linear salt gradient from 50 to 500 mM ammonium acetate buffer (pH 6), followed by step-wise elution at 1000 mM. Active fractions were concentrated with an Amicon ultracentrifugal filter membrane (protein cut off of 10 kDa) for further analysis.

4.2.3 Results and discussion

The elution profiles of Superdex 200 HR chromatography of fractions AST1c and AST1d are shown in Figure 4.20.

(A)   (B)
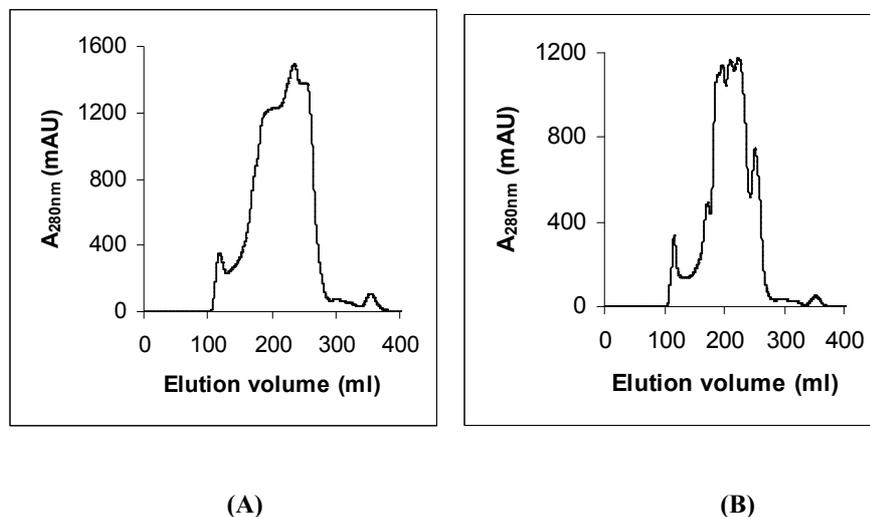
Figure 4.20: Superdex 200 HR chromatography of fractions (A) AST1c (1163.1 mg) and (B) AST1d (523.4 mg); Column: 2.6 x 60 cm); Tubes (2 ml each) were pooled (A): 53-65 (AST1cS1); 66-105 (AST1cS2); 106-125 (AST1cS3); 126-145 (AST1cS4); 146-170 (AST1cS5) and 171-188 (AST1cS6). Tubes (2 ml each) were pooled (B): 53-60 (AST1dS1); 75-90 (AST1dS2); 91-105 (AST1dS3); 106-120 (AST1dS4); 121-125 (AST1dS5); 126-145 (AST1dS6); 146-160 (AST1dS7) and 170-158 (AST1dS8). ─A_{280nm}.

Elution profiles of HiTrap SP chromatography of fractions AST1cS2 and AST1dS4 are shown in Figure 4.21.

(A)   (B)
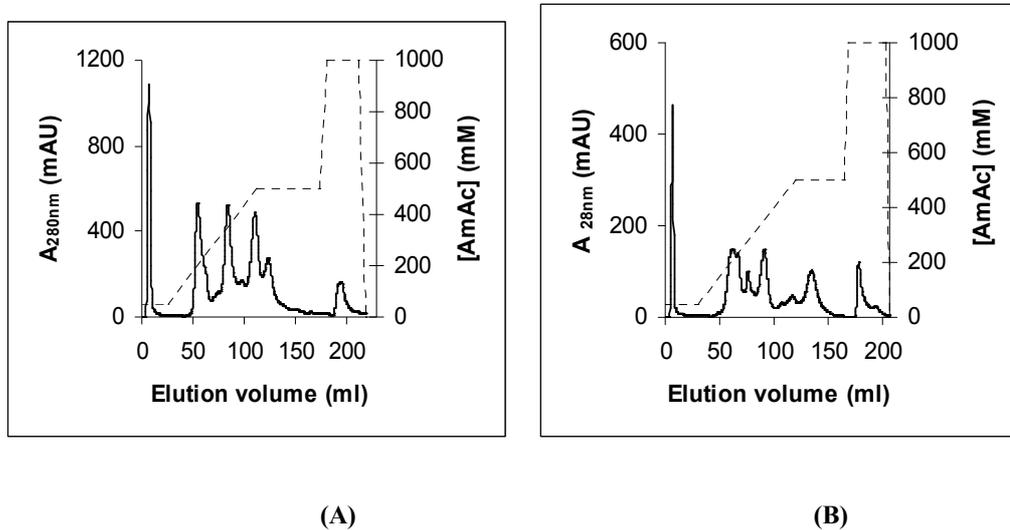
Figure 4.21 HiTrap SP HR chromatography of (A) AST1cS2 (153mg) and (B) AST1dS4 (97 mg). Column: 1.6 x 2.6 cm. Linear salt gradient: 50-500mM ammonium acetate buffer (pH 6). Tubes (2 ml each) were pooled (A): 3-8 (AST1cS2SP1); 25-32 (AST1cS2SP2); 33-36 (AST1cS2SP3); 37-39 (AST1cS2SP4); 41-49 (AST1cS2SP5); 50-60 (AST1cS2SP6); 61-77 (AST1cS2SP7); 87-94 (AST1S2SP8). Tubes (2 ml each) were pooled (B): 4-9 (AST1dS4SP1); 25-26 (AST1dS4SP2); 27-30 (AST1dS4SP3); 34-43 (AST1dS4SP4); 48-56 (AST1dS4SP5); 57-69 (AST1dS4SP6); and 91-100 (AST1dS4SP7). ─A$_{280nm}$; --- [AmAc].

A summary of the isolation and purification procedure of ostrich MBSPI is shown in Table 4.5

Table 4.5: Summary of the isolation and purification procedure of ostrich MBSPI from 1.83 kg minced skeletal muscle.

Purification step	Fraction code	Protein (mg)*	Inhibition (%)
Ammonium sulfate crude extract	AS	32000	47.3
Toyopearl Super Q 650S	AST1c AST1d	1163.1 523.4	50 40.3
Superdex 200	AST1cS2 AST1dS4	154.0 97	68.3 69.6
HiTrap SP HR	AST1cS2SP2 ASTIcS2SP3 AST1dS4SP2 AST1dS4SP5	17.517 4.778 1.932 1.843	100 100 100 100

*Not all proteins recovered from the Toyopearl Super Q 650S batch-wise purification step (fraction AST1) have been used. Only approximately 2 g of lyophilized fraction AST1 was used for this study. Hundred (100%) inhibition was detected using reverse zymogram, as shown in Figures 4.23 and 4.24.

SDS-PAGE patterns of fractions from HiTrap SP HR chromatography (partially purified MBSPI fractions AST1dS4SP2 and AST1dS4SP5) are shown in Figure 4.22.

(A)   (B)
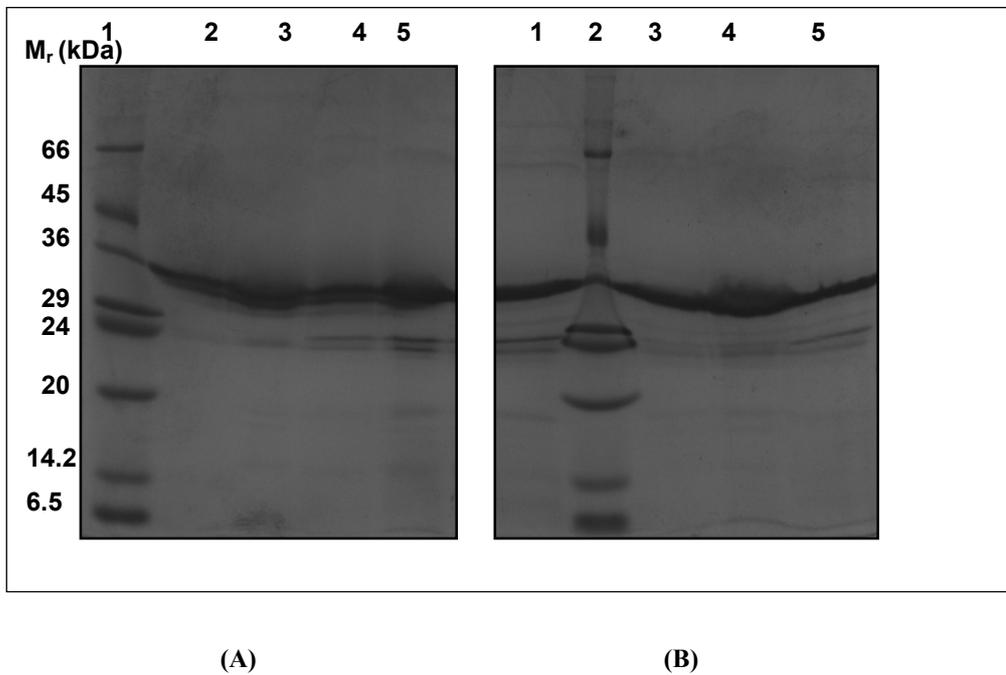
Figure 4.22 SDS-PAGE patterns of fractions from HiTrap SP HR chromatography. Under (A) non-reducing conditions. Lanes: 1, LMW markers; 2, 10 µg AST1dS4SP2; 3, 20 µg AST1dS4SP2; 4, 10 µg AST1dS4SP5; 5, 20 µg AST1S4dSP5. (B) reducing conditions. Lanes: 1, 20 µg AST1S4dSP5; 2, LMW markers; 3, 10 µg AST1dS4SP2; 4, 20 µg AST1dS4SP2; 5, 10 µg AST1dS4SP5.

Reverse zymography of fractions obtained from HiTrap SP HR chromatography (Figure 4.21B) is shown in Figure 4.23.
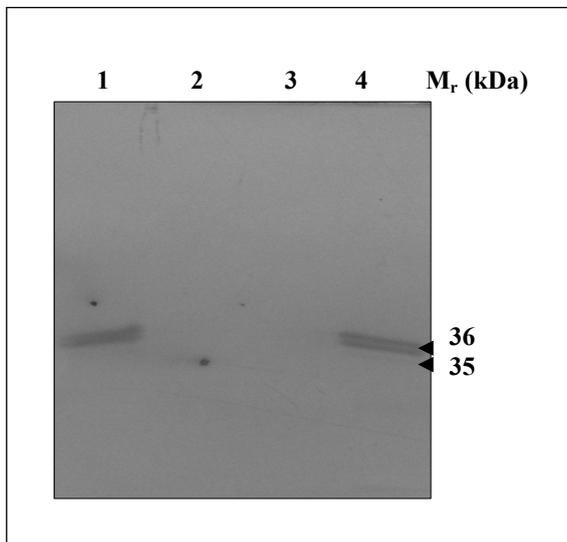
Figure 4.23 Reverse zymography of fractions obtained from HiTrap SP HR chromatography (Figure 4.21B). Fractions 20µg/lane were loaded. Lanes: 1, AST1dS4SP2; 2, AST1dS4SP3; 3, AST1dS4SP4 and 4, AST1dS4SP5.

Reverse zymography of fractions obtained from HiTrap SP HR chromatography (Figure 4.21A) is shown in Figure 4.24.
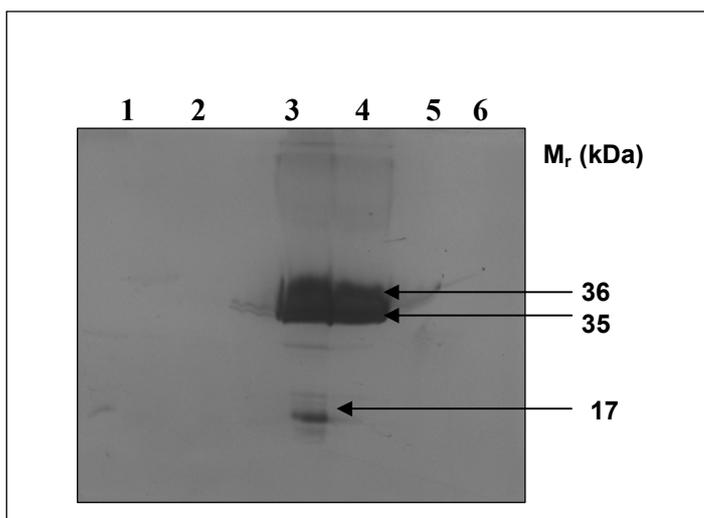
Figure 4.24. Reverse zymography of fractions obtained from HiTrap SP HR chromatography (Figure 4.21A). Fractions (20µg/lane) were loaded. Lanes: 3, AST1cS2SP2; 4, AST1cS2SP3; 5, AST1cS2SP4 and 6, AST1cS2SP5.

Ostrich MBSPIs were partially purified by ammonium sulfate fractionation followed by three chromatographic steps, namely Toyopearl Super Q, Superdex 200 and HiTrap SP. The inhibitors showed a strong interaction with the HiTrap SP resin and eluted with a linear AmAc salt gradient buffer (Figure 4.21). The partially purified MBSPIs were eluted at 200 mM AmAc buffer (pH 6) as a single peak, fractions AST1cS2SP2 and AST1cS2SP3; (Figure 4.21A), while it eluted at 200 and 350mM AmAc buffer (pH 6) as single peaks, fractions AST1dS4SP2 and AST1dS4SP5 (Figure 4.21B). The purification results of MBSP inhibitors are summarized in Table 4.5. The yield obtained in this purification procedure is comparable to the yield of protease M endogenous inhibitor from mouse, which was 0.39 mg protein from pooled mice skeletal muscle (25 g x 14), revealing a single band of M_r 110 kDa under reducing and non-reducing conditions (Sangorrin *et al.*, 2002). Inhibitory activity peaks against bovine trypsin could be detected using reverse zymography (Figures 4.23 and 4.24), revealing bands of M_r 17, 35 and 36 kDa.

The purity and M_r of MBSPI fractions AST1dS4SP2 and AST1dS4SP5 (Figure 4.21B) were analyzed on SDS-PAGE using various protein concentrations (Figure 4.22). Ostrich MBSPIb revealed 2 major bands with approximately M_r values of 35 and 36 kDa under non-reducing conditions and a single band of 36 kDa under reducing conditions for both fractions, indicating that the inhibitors resolved from different separated peaks are identical (Figures 4.22 and 4.23). However, fractions AST1cS2SP2 and AST1cS2SP3 were pooled from a single peak, where fraction AST1cS2SP2 was from the ascending part of the peak and AST1cS2SP3 from the descending part of the peak. Fraction AST1cS2SP2 have 2 inhibitors resolved with different M_r values of 17 and 35/36 kDa, of which M_r 35 and 36 kDa bands are identical to M_r values revealed from fractions AST1dS4SP2 and AST1dS4SP5 (Figures 4.22 and 4.23), respectively. The partially purified MBSPs were subjected to SDS-PAGE, transferred to PVDF membranes and sent to Prof. Muramoto for N-terminal sequence analysis.

Cao *et al*. (2001) obtained 3.3 mg of purified MBSPI from 0.5 kg skeletal muscle of lizard fish, revealing an M_r of 50 kDa using SDS-PAGE and SEC. Sangorrin *et al*. (2001) obtained 0.6 mg of purified MBSPI from 0.3 kg of white croaker skeletal muscle,

revealing an M_r of 65 kDa using SDS-PAGE and SEC. All purified endogenous inhibitors were monomeric as analyzed by SDS-PAGE.

CHAPTER 5: PARTIAL CHARACTERIZATION OF OSTRICH MBSP AND ITS ENDOGENOUS INHIBITOR

5.1 PHYSICOCHEMICAL CHARACTERIZATION OF OSTRICH MBSP AND MBSPI

5.1.1 Molecular weight

The M_r determination of partially purified ostrich MBSP and MBSPIs were made using SDS-PAGE (12.5 and 13.5% gels), respectively. The M_r of ostrich MBSP was determined to be 21 kDa under non-reducing conditions (Figures 4.8 and 4.9). The M_r of ostrich MBSP is quite small compared to those purified from ordinary and skeletal muscles of various fish species and mouse, ranging from 28-120 kDa (Table 1.1), falling in the lower range of trypsin enzymes, that is 20-25 kDa. Guo *et al.* (2007) estimated the M_r of crucian carp (*Carassius auratus*) MBSP by SDS-PAGE and from the nucleotide sequence to be 25 and 28 kDa (monomer), respectively. Cao *et al.* (2000a) estimated the M_r of lizard fish (*Saurida wanieso*) MBSP by SDS-PAGE to be 60 and 29 kDa (homodimer). The M_r of carp fish (*Cyprinus carpio*) MBSP was estimated to be 30 kDa (monomer) by SDS-PAGE and gel filtration (Osatomi *et al.*, 1997). The M_r values of silver carp and mouse MBSPs were estimated to be 28 and 120 kDa (monomers) by gel filtration, respectively (Cao *et al.*, 2005; Sangorrin *et al.*, 2002). The M_r of lizard fish (*Saurida undosquamis*) MBSP was estimated to be 50 and 28 kDa (homodimer) by SDS-PAGE (Ohkubo *et al.*, 2004a). Ohkubo *et al.* (2005) estimated the M_r of white croaker (*Argyrosomus argentatus*) MBSP to be 67 kDa (monomer) by SDS-PAGE. These results show that the M_r values of MBSPs differ between individual species.

The M_r values of ostrich MBSPIs were estimated by SDS-PAGE to be 17 (monomer), 35 and 36 kDa (isomers) (Figure 4.24). These inhibitors are of low M_r in comparison to those purified from the skeletal muscle of fish species and mouse, ranging from 50-110 kDa (Table 1.2) and were all found to be monomers. However, the M_r of partially purified ostrich MBSPIs could be related to the low M_r of its target enzyme. Cao *et al.* (2000b) estimated the M_r of white croaker (*Argyrosomus argentatus*) MBSPI to be 55

kDa by SDS-PAGE. The M_r values of lizard fish (*Saurida wanieso*) and white croaker (*Micropogon opercularis*) MBSPI were estimated to be 50 and 65 kDa by SDS-PAGE and gel filtration, respectively (Cao *et al*., 2001; Sangorrin *et al*., 2001). Sangorrin *et al*. (2002) on the other hand, estimated the M_r of mouse MBSPI to be 110 kDa by gel filtration.

5.1.2 Substrate specificity

Substrate specificity of ostrich MBSP (fraction ST6AB2) and commercial bovine trypsin is shown in Table 5.1

Table 5.1 Summary of substrate specificities of ostrich MBSP and commercial bovine trypsin towards methyl-coumaryl-7-amide (MCA) synthetic substrates

Substrate (10 µM)	Ostrich MBSP (Relative activity) (%)	Commercial bovine trypsin (Relative activity) (%)	Crucian carp MBSP (Relative activity) (%)*
Boc-Phe-Ser-Arg-MCA	100	100	100
Boc-Val-Pro-Arg-MCA	356	258	123
Boc-Gln-Ala-Arg-MCA	382	196	-
Boc-Leu-Ser-Thr-Arg-MCA	53	43	-
Boc-Val-Leu-Lys-MCA	1	3	28
Suc-Leu-Tyr-MCA	0	0	0

*Guo *et al*. (2007)

Ostrich MBSP and commercial bovine trypsin rapidly hydrolyzed Boc-Phe-Ser-Arg-MCA, Boc-Val-Pro-Arg-MCA and Boc-Gln-Ala-Arg-MCA (Table 5.1). This is due to the enzyme's specificity toward an arginine residue at the P1 position of the substrates. However, ostrich MBSP showed highest specificity towards Boc-Gln-Ala-Arg-MCA containing alanine and glutamine residues at P2 and P3 positions, respectively, indicating that either of the two residues could also play a role in the enzyme's specificity. Commercial bovine trypsin showed highest specificity toward Boc-Val-Pro-Arg-MCA, containing proline and valine residues at P2 and P3 positions, respectively, which was also rapidly hydrolyzed by purified ostrich MBSP. Osatomi *et al.* (1997) suggested that

amino acid residue at P3 may affect the enzyme activity. A similar result was observed with Boc-Leu-Ser-Thr-Arg-MCA, containing arginine at the same position, showing 53 and 43% relative activity for ostrich MBSP and bovine trypsin, respectively. In comparison with Boc-Phe-Ser-Arg-MCA, Boc-Leu-Ser-Thr-Arg-MCA is a poor substrate (Table 5.1). On the other hand, both enzymes showed a very low hydrolysis of Boc-Val-Leu-Lys-MCA containing lysine at the P1 position, compared with arginine. The enzymes also showed no hydrolysis of the chymotrypsin substrate Suc-Leu-Tyr-MCA (Table 5.1). These results indicate that ostrich MBSP is an arginine-specific trypsin-like serine protease.

The results in this study are also comparable to MBSP from ordinary and skeletal muscles of various fish species. These results are similar to that obtained by Ohkubo *et al.* (2004a); purified lizard fish MBSP could not hydrolyze the same substrate used in this study containing a lysine residue at the P1 position and showed no hydrolysis of the chymotrypsin substrate, indicating that it is also an arginine-specific trypsin-like serine protease. It also showed 89% relative activity toward Boc-Val-Pro-Arg-MCA (Okhubo *et al.*, 2004a). Yellow croaker (*Pseudosciaena crocea*), crucian carp fish (*Carausius auratus*) and carp fish (*Cyprinus carpio*) MBSP, on the other hand, showed highest activity towards Boc-Val-Pro-Arg-MCA and they hydrolyzed various substrates containing arginine at the P1 position. These enzymes also showed specificity toward Boc-Val-Leu-Lys-MCA, as well as various substrates containing lysine at the same position. However, these enzymes could not hydrolyze various substrates for chymotrypsin, cathepsin L and B, iminopeptidase and metalloproteases, indicating that the purified enzymes are trypsin-like serine proteases (Cao *et al.*, 2006a; Osatomi *et al.*, 1997; Guo *et al.*, 2007).

5.1.3 pH optimum

The pH optimum profiles of ostrich MBSP (fraction ST6AB2) and commercial bovine trypsin are illustrated in Figure 5.1.
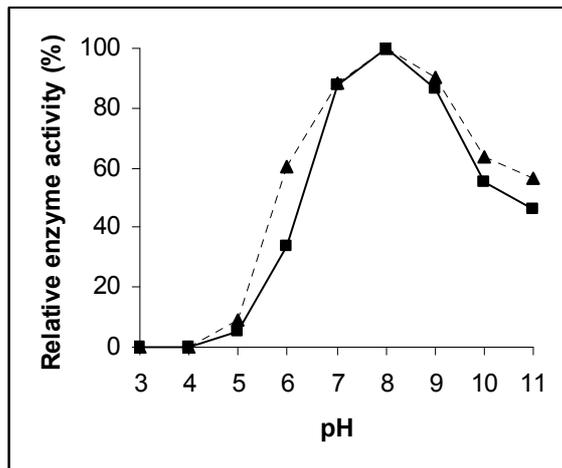
Figure 5.1: Effect of pH on ostrich MBSP and commercial bovine trypsin activities. Activity was measured in a series of buffers using 50 μM Boc-Phe-Ser-Arg-MCA as a substrate at 40˚C. Relative activity was estimated based on the highest enzyme activity taken as 100%. ▪ ostrich MBSP; --▲-- commercial bovine trypsin.

Ostrich MBSP and commercial bovine trypsin showed similar pH profiles since they retained more than 80% of the activity between pH 7.0 and 9.0, both having a pH optimum of 8.0. The activity of both enzymes decreased above pH 9.0 due to denaturation of enzymes. The decrease in activity of both enzymes was also observed at acidic pH values. Therefore, ostrich MBSP is considered an alkaline serine proteinase. The optimum pH value of ostrich MBSP is comparable to that of purified and partially purified serine proteases from white croaker skeletal muscle (Yanagihara *et al.*, 1990) that were most active at pH 8 and 9 using Boc-Phe-Ser-Arg-MCA, with an optimum pH of 8.0 at 38˚C. On the other hand, caseinolytic activity of the purified enzyme showed a maximum at pH 8.0 and 9.0, while for the partially purified enzyme the highest activity was at pH 8.0 at 60˚C (Yanagihara *et al.*, 1990). The optimum pH of ostrich MBSP was also similar to that of MBSP obtained from the skeletal muscle of yellow croaker (*Pseudosciaena crocea*) and crucian carp (*Caraussius auratus*) with an optimum pH of 8.0 at 50˚C, using the same substrate used for this study (Cao *et al.*, 2006a; Guo *et al.*,

2007). The purified enzymes retained activity above 80% at pH values between 7.5 and 8.5 and were inactivated at pH value above 9.0 and at acidic pH values, indicating that the functional pH of yellow croaker and carp MBSPs is in the alkaline range (Cao *et al*., 2006a; Guo *et al.,* 2007). Osatomi *et al*. (1997) obtained an optimum pH for purified MBSP from carp (*Cyprinus carpio*) ordinary muscle of 8.0 using Boc-Phe-Ser-Arg-MCA and casein.

Cao *et al*. (2005) on the other hand, obtained an optimum pH of 8.5 for purified MBSP from the skeletal muscle of silver carp using the same substrate used in this study. Purified MBSP from the skeletal muscle of lizard fish (*Saurida wanieso*) showed an optimum pH from 7.0 to 8.0 using Boc-Phe-Ser-Arg-MCA and the enzyme retained activity above 80% at pH values between 7.0 and 8.5 (Cao *et al*., 2000a). Purified MBSP from crucian carp muscle (*Carassius giberio langsdorfi*) was most active at pH values between 7 and 8 with Boc-Phe-Ser-Arg-MCA and between 8.0 and 9.0 with casein (Ohkubo *et al*., 2004b). Ohkubo *et al*. (2004a) obtained an optimum pH of 9.0 for purified MBSP from lizard fish (*Saurida undosquamis*) muscle using both Boc-Phe-Ser-Arg-MCA and casein. These enzymes were denatured at pH above 9.0 and inactivated at acidic pH.

The optimum pH of commercial bovine trypsin (Figure 5.1) is comparable to that of trypsin from the pyloric caeca of Monterey sardine *Sardinops sagax caerulea*, with an optimum pH of 8.0, using a trypsin specific substrate BAPNA, with the activity decreasing with increasing pH (Castillo-Yáñez *et al*., 2005) .

5.1.4 Temperature optimum

The temperature optimum profiles of ostrich MBSP (fraction ST6AB2) and commercial bovine trypsin are illustrated in Figure 5.2.
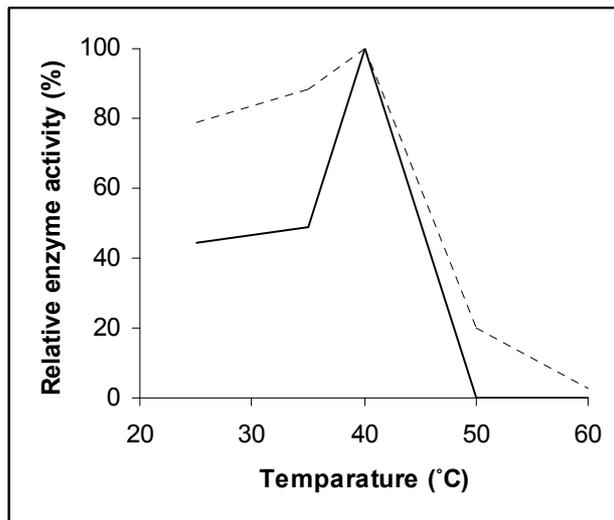
**Figure 5.2**: Effect of temperature on ostrich MBSP and commercial bovine trypsin activities. Activity at pH 8 (100 mM Tris/50 mM histidine/ 100 mM sodium acetate buffer) was evaluated using 50 µM Boc-Phe-Ser-Arg-MCA. Relative enzyme activity was estimated based on the highest activity taken as 100%. ─ ostrich MBSP; ---- commercial bovine trypsin.

Ostrich MBSP and commercial bovine trypsin showed a temperature optimum of 40˚C at pH 8.0. Ostrich MBSP is more inactivated below and above 40˚C compared to commercial bovine trypsin. However, at 50˚C ostrich MBSP is inactivated similar to commercial bovine trypsin, retaining 0 and 20% activity, respectively, due to thermal denaturation. The optimum temperature of ostrich MBSP is comparable to that of a purified serine protease from white croaker muscle, with an optimum temperature of 40˚C using Boc-Phe-Ser-Arg-MCA and casein at pH 8.0, while the partially purified serine protease showed temperature optima of 50 and 60˚C, respectively (Yanagihara *et al*., 1991). These enzymes retained 50% and more activity towards Boc-Phe-Ser-Arg-MCA at temperatures below 40˚C and no caseinolytic activity (Yanagihara *et al*., 1991).

In terms of temperature ostrich MBSP is also comparable to crude, partially purified and highly purified MBSPs from ordinary and skeletal muscles of various fish species. The

crude MBSP from yellow croaker (*Pseudosciaena crocea*) muscle showed an optimum temperature of 55°C, while partially purified enzyme showed an optimum temperature of 45°C at pH 8.0, using the same substrate used in this study. Purified enzyme retained more than 80% activity at temperatures between 35 and 50°C, and more than 60% activity at 30 and 55°C. It also retained about 25% activity at 25 and 60°C, and no activity at 70°C due to thermal degradation. On the other hand, crude enzyme retained 90% activity at 50°C, while at 45 and 60°C, 60% activity was retained and less than 40% activity at temperature between 25 and 40°C and at 70°C, respectively (Cao *et al.*, 2006a). The partially purified silver carp MBSP showed an optimum temperature of 55°C at pH 8.0 using the same substrate as used for this study. The enzyme retained more than 80% activity towards Boc-Phe-Ser-Arg-MCA at temperatures between 50 and 60°C, while showing very low activity at 70°C, indicating that the enzyme was denatured (Cao *et al.,* 2005).

The highly purified MBSP from carp (*cryprinus carpio*) muscle showed an optimum temperature of 55°C at pH 8, the same as partially purified silver carp MBSP using Boc-Phe-Ser-Arg-MCA as a substrate. The enzyme retained 80% activity at 60°C, and 60% activity at 50 and 70°C, while it retained 40 and 50% activity at 30 and 40°C, respectively. The enzyme also retained 20% activity at 25 and 70°C (Guo *et al.*, 2007). The purified MBSP from lizard fish (*Saurida wanieso*) muscle showed an optimum temperature of 50°C using the same substrate as used for this study. The enzyme retained more than 80% activity at temperatures between 40 and 55°C, while at temperatures between 35 and 45°C, 45 and 65% activity was retained. It also retained 20 and 25% activity at 30 and 65°C, respectively, and the enzyme was denatured at 65°C, retaining less than 55% activity (Cao *et al.*, 2000a).

The optimum temperature of commercial bovine trypsin (Figure 5.2) was comparable to trypsin from pyloric caeca of Monterey sardine (*Sardinops sagax caerulea*), which showed an optimum temperature of 50°C using BAPNA as a substrate. The enzyme retained more than 80% activity at temperatures between 40 and 50°C, while retaining more than 40% activity between 30 and 35°C, and less than 40% activity was retained at

10, 20, and 50°C, and was denatured at 60°C, with less than 25°C activity retaining (Castillo-Yáñez *et al.*, 2005),

5.1.5 N-terminal amino acid sequence analysis

5.1.5.1. N-terminal amino acid sequence of MBSP

M_r components of 21 and 22 kDa from ostrich MBSP (fraction ST6AB2) and commercial bovine trypsin, respectively, were subjected to N-terminal amino acid sequence analysis in the laboratory of Prof. Koji Muramoto, Sendai, Japan. Sequences aligned with other mature trypsin- type serine proteases, as well as hamster chymotrypsin-type serine proteases as shown in Table 5.2.

Table 5.2: Comparison of the N-terminal sequence of ostrich MBSP with sequences from other mature trypsin-type serine proteases as well as hamster chymotrypsin-type serine protease.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
OMBSP			A	D	Y	E	V	G	A	N	V										
CBT	I	V	G	G	Y	T	C	G	A	N	T	V	P	Y	Q	V	S	L	N	S	G
OT[†]	I	V	G	G	Y	N	C	P	A	H	S	V	P	Y	Q	V	S	L	N	S	G
LMBSPu[‡]	I	V	G	G	Y	E	X	E	A	Y	S	K	P	Y	Q	V	S	I	N	L	G
LMBSPw[‡]	I	V	G	G	A	E	X	V	P	Y						V	S	L	N	S	G
CMBSP[‡]	I	I	G	G	A	E	X	K	P	H	S	Q	P	W	Q	A	F	L	V	D	N
TCA[‡]	I	V	G	G	Y	E	X	E	P	Y	S	T	P	W	Q	A	S	L	N	S	G
TCB[‡]	I	V	G	G	Y	E	X	T	P	H	S	Q	P	W	Q	V	S	L	N	S	G
TS[‡]	I	V	G	G	Y	E	C	K	A	Y	S	Q	P	H	Q	V	S	L	N	S	Y
TC[‡]	I	V	G	G	Y	E	C	T	R	H	S	Q	A	H	Q	V	S	L	N	S	Y
CCMBSP*	I	V	G	G	Y	E	C	R	P	H	S	Q	P	W	Q	A	F	L	T	D	N
PT*	I	V	G	G	Y	T	C	A	A	N	S	I	P	Y	Q	V	S	L	N	S	G
HM*	M	T	A	A	H	C	G	R	P	I	L	Q	V	A	L	G	K	H	N	L	C

[†]Szenthe *et al.* (2005); [‡]Ohkubo *et al.* (2004a) (see Figure 1.4); * Guo *et al.* (2007). OMBSP, ostrich MBSP; CBT, commercial bovine trypsin; OT[†], ostrich trypsin; LMBSPu[‡], lizard fish (*Saurida undosquamis*) MBSP; LMBSPw[‡], lizard fish (*Saurida wanieso*) MBSP; CMBSP[‡], carp MBSP; TCA[‡], trypsin carp A; TCB[‡], trypsin carp B; TS[‡], trypsin salmon; TC[‡], trypsin cod; CCMBSP*, crucian carp MBSP; PT*, porcine trypsin and HM*, hamster mekratin , a chymotrypsin-type serine protease. Identical amino acid residues to that of ostrich MBSP are highlighted.

Ostrich MBSP revealed a very short N-terminal sequence of 9 amino acid residues, while for commercial bovine trypsin a longer sequence of 21 amino acid residues is shown (Table 5.2). Ostrich MBSP showed some identity (4 residues) to bovine trypsin, and 3 identical residues to lizard fish MBSP (*Saurida undosquamis*), porcine trypsin and salmon trypsin. It showed low identity (2 residues) to ostrich trypsin, carp fish MBSP, carp trypsin A and B, and cod trypsin. However, very low identity (1 residue) was observed with carp MBSP, lizard MBSP (*Saurida wanieso*) and hamster mekratin, suggesting ostrich MBSP is a trypsin-like serine protease.

5.1.5.2 N-terminal amino acid sequences of MBSPIs

Ostrich MBSPIa and MBSPIb (fractions AS and AST1dS4SP5) with bands corresponding to M_r values 17, 35 and 36 kDa were blotted separately to PVDF membranes and their N-terminal amino acid sequences analyzed. The N-terminal amino acid sequences of MBSPIs are shown in Tables 5.3 and 5.4.

Table 5.3: Comparison of the N-terminal sequence of ostrich MBSPI (M_r 17 kDa) with sequences from lizard MBSPI, as well as other trypsin inhibitors (Kunitz family) and horse heart myosin.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
OMIa	G	L	S	D	Q	E	W	Q	Q	V	L	T	I	W	G	K	V	E	S	D	I
OM†	G	L	S	D	Q	E	W	Q	Q	V	L	T	I	W	G	K	V	E	S	D	I
HM†	G	L	S	D	G	E	W	Q	Q	V	L	N	V	W	G	K	V	E	Q	V	L
LMI‡		L	Q	D	W	Y	D	N	T	H	H	G	Y	L	G	K	D	T	S	E	V
WTI*	P	L	L	D	S	E	G				L	V	R	N	G	T	Y	Y	L	L	P
PTI*	P	L	E	D	S						L	A			A	K	L	N			
ECI*			V	D	L	E	G	N	L	V	E	N		F	G						I
KTI*		L	Q	N	G	G	T	Y	Y	M	L										

†Miyazaki *et al*. (1998); ‡Cao *et al*. (2000b); *Garcia *et al*. (2004). OMIa, ostrich MBSP inhibitor; OM†, ostrich myoglobin; HM†, horse heart myoglobin; LMI‡, lizard fish MBSPI; WTI*, *Psophocarpus tetraganobus* trypsin inhibitor; PTI*, *Poecilanthe parviflora* trypsin inhibitor; ECI*, *Erythrina variegate* chymotrypsin inhibitor and KTI*, Kunitz soybean trypsin inhibitor. Identical amino acid residues with that of ostrich MBSPI (M_r 17 kDa) are highlighted.

The N-terminal amino acid sequence of ostrich MBSPIa (M_r 17 kDa) revealed 33 amino acid residues, of which 21 are shown (Table 5.3). The latter sequence showed 100% identity to myoglobin from ostrich (*Struthio camelus*) and 71% identity to myoglobin from horse heart (Miyazaki *et al*., 1998). However, the ostrich inhibitor showed 24% identity to lizard fish MBSPI, the latter showing 74% homology to porcine

phosphoglucose isomerase (PGI) (Cao *et al.*, 2001), which was found to suppress the activity of lizard fish MBSP. Ostrich MBSPIa showed 24% identity to a chymotrypsin-type serine protease inhibitor from *Erythrina variegate*. Ostrich MBSPIa showed 19 to 24% identity to trypsin inhibitors from legumes seeds of *Psophocarpus tetraganobus and Poecilanthe parviflora*, respectively, suggesting characteristics of a Kunitz-type trypsin inhibitor.

Table 5.4: Comparison of the N-terminal sequences of ostrich MBSPIs (M_r 17 and 35 kDa) with sequences from lizard fish MBSPI, as well as other trypsin inhibitors (Kunitz family) and mammalian glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
OMIa	G	K	V	E	S	D	I	A	G	H		G	H	A	I	L	M	R			L
OMIb	V	K	V	G	I	N	G	F	G	R	I	G	R	L	V	G	R	A	A	I	L
RMG*		K	V	G	V	N	G	F	G	R	I	G	R	L		T	R	A	A	F	N
HLG*		K	V	G	V	N	G	F	G	R	I	G	R	L	V	T	R	A	A	F	N
LMI†	L	Q	D	W	Y	D	N	T	H	H		G	Y	L	K	D	T	S	E	V	A
WTI‡	P	L	L	D	E	S	G	L	V	R	N	G	G	T	Y	Y	L	L	P	D	
PTI‡	A	P	L	E	S	L												A	A	K	L
ECI‡	V			D	L	E	G	N	L	V	E	N	F	G	T	Y	Y			I	L
KTIb‡														L	Q	N	G	G	T	Y	M

*Ren *et al.* (2005); †Cao *et al.* (2000b); ‡Garcia *et al.* (2004). OMIa, ostrich MBSPI (M_r 17 kDa); OMIb, ostrich MBSPI (M_r 35 kDa); RMG*, rat muscle GAPDH; HLG*, human liver GAPDH; LMI†**,** lizard fish MBSP inhibitor; WTI‡**,** *Psophocarpus tetraganobus* trypsin inhibitor; PTI‡, *Poecilanthe parviflora* trypsin inhibitor; ECI‡**,** *Erythrina variegate* chymotrypsin inhibitor; and KTI‡, Kunitz soybean trypsin inhibitor. Identical amino acid residues with that of ostrich MBSPI (M_r 35 kDa) are highlighted.

The N-terminal sequence of ostrich MBSPIb (M_r 35 kDa band) showed 100% identity to that of the M_r 36 kDa band, suggesting that they are the same protein (fractions AST1dS4SP2 and AST1dS4SP5) as detected in SDS-PAGE (Figure 4.22 and 4.23). Ostrich MBSPIa showed 19% identity to MBSPIb. Ostrich MBSPIb shared high identity (76%) to GADPH from rat muscle and human liver. Ostrich MBSPIb shared 14% identity to trypsin inhibitors from legume seeds of *Psophocarpus tetraganobus and Poecilanthe parviflora*, as well as 19% identity to a chymotrypsin inhibitor from *Erythrina variegate*, suggesting the characteristics of both trypsin and chymotrypsin-like serine proteases of Kunitz-type inhibitors (Table 5.4).

5.2 KINETIC CHARACTERIZATION OF MBSP

5.2.1 Kinetic parameters

Lineweaver-Burk plots for ostrich MBSP (fraction ST5AB2) and commercial bovine trypsin are shown in Figure 5.3.

(A)   **(B)**
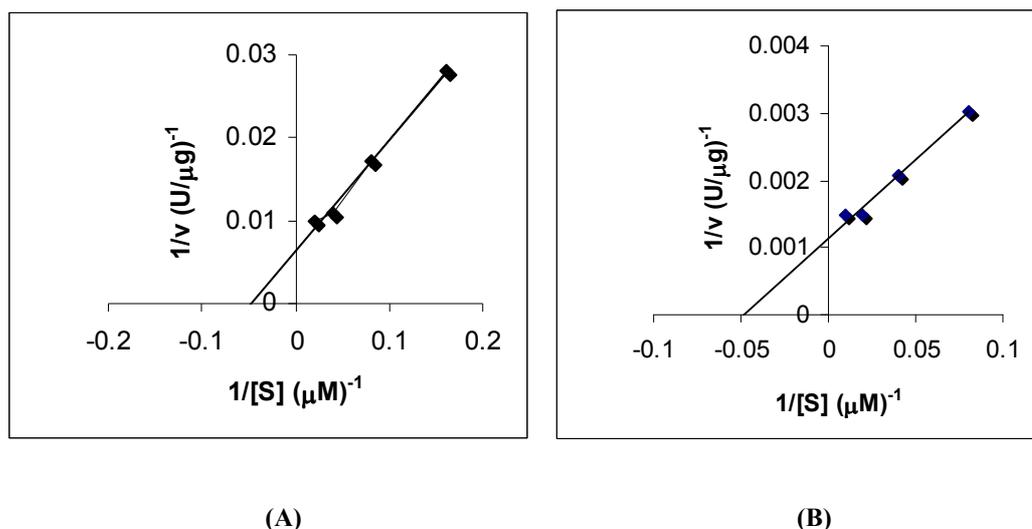
Figure 5.3: Lineweaver-Burk plots for (A) ostrich MBSP (r^2= 0.9921) and (B) commercial bovine trypsin (r^2= 0.9826). Boc-Val-Pro-Arg-MCA was used as a substrate.

A summary of Michaelis-Menten constant, K_m, and maximum velocity, V_{max}, values are given in Table 5.5.

Table 5.5: Summary of K_m and V_{max} values of ostrich MBSP and commercial bovine trypsin

Enzyme	K_m (μM)	V_{max} (U/μg)	V_{max}/K_m (U/μg/μM)
Purified ostrich MBSP	20.8	153.8	7.4
Commercial bovine trypsin	21.4	909.1	42.5

The K_m and V_{max} values obtained for purified ostrich MBSP and commercial bovine trypsin were estimated to be 20.8 and 21.8 μM, and 153.8 and 909.1 U/μg, respectively. The K_m value obtained for purified ostrich MBSP is comparable to that of carp (*Cyprinus carpio*) MBSP (Cao *et al.*, 1999b), which was estimated to be 6.7 μM using the same

substrate used in this study, indicating that carp MBSP has a higher affinity for Boc-Val-Pro-Arg-MCA than ostrich MBSP. Fukusen and Aoki (1996) determined K_m values of novel trypsin-like serine proteases to be 3.53-5 µM using the same substrate used in this study, indicating their high affinity for the substrate. On the other hand, K_m values of carp MBSP obtained by Cao *et al.* (1999b) for various MCA-substrates ranged from 6.7 to 79.6 µM. The V_{max} and V_{max}/K_m values of these substrates were not reported. Ostrich MBSP showed a 5.7 times lower specificity constant compared to bovine trypsin. The trypsin K_m values described in a review by Castillo-Yáñez *et al.* (2005) from various fish species ranged from 29 to 660 µM using BAPNA as a substrate.

CHAPTER 6: FINAL CONCLUSIONS

The majority of the objectives of this study as outlined in Chapter 2 were achieved. Ostrich MBSP was partially characterized. The characterization of MBSPI was limited to those physicochemical aspects presented in section 5.1.2; therefore, the necessary kinetic and physicochemical characteristics of the partially purified MBSPI could not be reported.

Four isolation and purification attempts of ostrich MBSP were undertaken and compared with various other studies for the purification of this enzyme from fish species and mouse. The second isolation attempt successfully dissociated MBSP from myofibrils using a method of Sangorrin *et al.* (2002) and the enzyme was partially purified using Toyopearl Super Q 650S and p-aminobenzamidine-Agarose chromatographies. The functional characterization of ostrich skeletal muscle MBSP obtained from the second isolation and purification attempt included optimum pH and temperature, and kinetic studies. Ostrich MBSP showed many similar physicochemical characteristics to MBSP from lizard fish, white croaker and yellow croaker muscle, as well as from muscle of other fish species and mouse, but was substantially different for its optimum temperature. Characteristics of isolated ostrich MBSP are interesting from a technological prospective, especially maximum activity at pH 8.0, optimum temperature at 40˚C and specific cleavage at the carboxyl side of arginine residues of synthetic fluorogenic substrates. The kinetic properties of ostrich MBSP are very similar to those obtained for commercial bovine trypsin, indicating that it is a trypsin-like serine protease. These characteristics suggest that ostrich MBSP could be an important biotechnological tool for ostrich meat processing and food industries.

Ostrich skeletal muscle was found to be a rich source of trypsin-like inhibitors. These inhibitors were also found to be located on myofibrils, suggesting that they might form a complex with MBSP and its unknown natural substrate, resulting in high M_r component(s) as observed in Figures 4.7 (lanes 6 and 7) and 4.19 (lane 4). Based on SDS-PAGE results, it was shown that after passing an MBSP fraction through a p-aminobenzamidine-Agarose column, a complex could be dissociated, resulting in low M_r

components, corresponding well with those detected for the partially purified ostrich MBSPIs (Figure 4.8, lane 3, compared to Figures 4.22, lanes 1-4, and 4.24, lanes 3-4), as well as of purified ostrich MBSP, suggesting that these binding patterns caused difficulties in purifying ostrich MBSP to homogeneity. Furthermore, an investigation is needed on the binding pattern of ostrich MBSP to myofibrils in order to identify an exact myofibrillar protein target of the enzyme. The action of ostrich MBSP on myofibrillar proteins is of interest for further studies.

For ostrich MBSPI one isolation and purification attempt was reported in this study, though several chromatographies were attempted, resulting in negative results and they could not be reported. Ostrich MBSPIs were fractionated from a sarcoplamic fraction resulting from the fourth isolation and purification attempt of MBSP, according to Cao *et al*. (2001) (section 4.1.4.1). MBSPIs were fractionated by 75% ammonium sulfate saturation and via three chromatographic steps, involving Toyopearl Super Q, Superdex 200 and finally HiTrap SP HR. Partially purified ostrich MBSPI bands were quantified on casein reverse zymography, revealing M_r values of 35 and 36 kDa, respectively, from fractions AST1dS4SP2 and AST1dS4SP5 resulting from HiTrap SP HR chromatography (Figure 4.21B). These fractions were transferred to PVDF membranes (similar SDS-PAGE patterns shown in Figures 4.22 and 4.23) under reduced and non-reduced conditions and sent for N-terminal sequence analysis by Prof. Koji Muramoto. Fractions AST1dS4SP2 and AST1dS4SP5 showed identical N-terminal amino residues under non-reducing conditions, suggesting that they are the same protein. Surprisingly, one ostrich MBSPI sequence (M_r 35 kDa) shared high sequence identical (74%) to GAPDH from human and rat. The biological significance of the high identity between ostrich MBSPI and GAPDH could be of interest for further studies.

One ostrich MBSPI form revealed an M_r of 17 kDa from fraction AST1cS2SP2 (Figure 4.24). AS fraction (crude MBSP fraction) with an M_r corresponding to 17 kDa was transferred to PVDF membranes and sent for N-terminal sequence analysis, showing 100% identity to the ostrich myoglobin sequence. The biological significance of the latter identity could be of interest for further studies. The distribution of MBSPIs in ostrich tissues could also be investigated for further studies.

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