2002

Permeability of rice cystatin across Caco-2 cells

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PERMEABILITY OF RICE CYSTATIN ACROSS CACO-2 CELLS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Food Science

by
Tania Maria Serigado Antunes
B.S., Superior School of Biotechnology, Portuguese Catholic University, Portugal, 1998
December 2002

ACKNOWLEDGEMENTS

The author would like to express her gratitude and thanks to her major advisor, Dr. Jack N. Losso, Assistant Professor, Department of Food Science, for his assistance and guidance throughout the completion of this work.

She is grateful to her graduate committee composed of Dr. Terry Walker, and Dr. Fred Shih for their assistance, and valuable comments and suggestions.

Acknowledgments are due to Dr. Masahiro Ogawa for giving the author a closer guidance and suggestions throughout the completion of this work, as well as Dr. Robert Truax for providing the author with the Caco-2 cells and letting the author use his laboratory facilities for the completion of the work.

The financial support of the Louisiana Rice Research Board, which made the present work possible, and covered the author's assistantship, is greatly acknowledged.

Special acknowledgments go to my friends Annie, Armen, Cate, and Daniel at LSU for their true inspiration and help through the darkest of times. Fortunately, the author has many friends to thank, not only for their invaluable help in overcoming difficult moments, but in some cases for their promptness in helping the author completing her thesis work. A special thanks goes to Rishipal and Armen for their assistance in the author's work, which made it possible and more enjoyable.

The author is especially grateful to her parents Diodete, who was always present in thoughts and gave the author tremendous encouragement and moral support, and Manuel Fernando. The author would like to thank her sisters Claudia and Liliana for their

invaluable help in difficult moments, her grandparents Joao and Joaquina, for their encouragement and moral support, and her brother-in-law, Mane.

Finally, a very heartfelt gratitude goes for Dr. Nehal Mohamed, the person that encouraged the author to pursue her studies and achieve this goal. To the author's mentor in life, this work is dedicated.

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ABSTRACT

The present work aimed at recovering, purifying, and testing rice cystatin or oryzacystatin I (OCI) bioavailability, to provide scientific evidence that rice cystatin can be used as a functional food ingredient. OCI was extracted from rice bran using 25 mM sodium phosphate buffer containing 0.15 M NaCl at pH 7.0. The resulting homogenate was heat treated, cooled and precipitated with ammonium sulfate. The recovered protein was dialyzed against 50 mM sodium acetate buffer pH 4.8 and sequentially purified by cation exchange, size exclusion, and anion exchange chromatography. Nine hundred and seventy micrograms of protein were obtained from 1 kg of rice bran. SDS-PAGE provided one pure band. MALDI-MS identified the protein at 11,538 Da. Overall there was a 14-fold increase in specific activity throughout the purification process.

OCI was hydrolyzed with chymotrypsin in 20 mM sodium phosphate buffer pH 7.4 for 2.5 hours at 37ºC at an enzyme to protein ratio of 1:100. The resulting chymotryptic peptides yielded a higher inhibitory activity compared to unhydrolyzed OCI (145% increase). The permeability of OCI and OCI chymotryptic peptides across Caco-2 cells was evaluated for 3 hours at 37ºC. Unhydrolyzed OCI did not cross the cell monolayer. OCI chymotryptic peptides were uptaken by the Caco-2 cells, as they were not detected in the apical side of the cells either by MALDI MS or papain inhibitory activity assay. After three-hour incubation, only one peptide with a molecular weight of 5.8 kDa was detected in the basolateral side of the cells. The peptide that crossed the

basolateral membrane had no inhibitory activity versus papain. The significance of these findings and future research direction will be discussed.

CHAPTER 1 INTRODUCTION

1.1 Functional Foods

1.1.1 Definitions of Functional Foods/Nutraceuticals

In the United States, DeFelice (1995) defined nutraceuticals / functional foods as "any substance that is food or part of a food and provides medical or health benefits, including the prevention and treatment of diseases". From the FDA's perspective, functional foods can fall into a number of existing categories of the Federal Food, Drug and Cosmetic Act of 1938 (FDCA), as amended. If the product is determined to be a food and not a drug, it can be regulated as conventional foods (including foods for special dietary use), dietary supplements, medical foods, or as infant formulas.

In Canada, two definitions emerged. A nutraceutical is "a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic diseases". A functional food "is similar in appearance to, or may be, a conventional food, is consumed as part of a usual diet, and is demonstrated to have physiological benefits and / or reduce the risk of chronic disease beyond basic nutritional functions" (Dentali, 2002).

In Japan, functional foods are "foods for specific health uses" (FOSHU). Functional foods in Japan have a history since the 1950s, and regulations towards its concept have been introduced (Dentali, 2002). In Japan, functional foods were based on the concept that food and drugs have the same origin (Hickling, 1997). Japan is the only country with a regulatory framework for functional foods (Dentali, 2002).

The European Commission Concerted Action on Functional Food Science states that "a food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction in risk of disease. Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet. They are not pills or capsules, but part of a normal food pattern." (Erkkila, 2002).

1.1.2 Rationale of the Emerging Need for Functional Foods

There is a demand for convenient, high quality, and healthy products. Some of the causes of these demands concern factors such as an aging population, highly health conscious consumers coupled with increase health care costs, self-efficacy and autonomy in health care and consumer dissatisfaction with certain aspects of the medical establishment. In addition, changes in food regulations, including the expanded category of dietary supplements, also play an important role in the development of functional foods. Also, the advance in scientific evidence that diet can alter disease prevalence and progression, including coronary heart disease and some cancers, play an important role in the development of functional foods too (Ghai, 2000; Milner, 2000; Kubena et al., 1999). People are becoming increasingly convinced that the foods they eat can influence their risk of acquiring a variety of diseases, apart from modulating performance (Wrick, 1995). Therefore, health foods, botanicals, functional foods, and nutraceuticals, satisfy this market demand (Ghai, 2000). The global functional foods market was estimated to be $47.6 billion in 2002 (Institute of Food Technologists Functional Foods Newsletter,

2002). The United States is the largest market segment at $18.25 billion. The Japanese market is at $11.8 billion (Institute of Food Technologists Functional Foods Newsletter, 2002).

1.2 Rice

1.2.1 Rice Production and Consumption

The world production of rice is approximately 500 million metric tons per year (Gingras et al., 2000). In the United States, the consumption of rice has doubled in more than a decade, at more than 21 pounds per capita. According to the California Rice Commission, rice consumption has been increasing at about 4% a year and rice authorities expect this trend to continue. One of the factors that contribute to this increasing trend is the consumer's interest in healthy diets (Pszczola, 2001).

1.2.2 Structure of Rice Grain

The rice grain consists of two distinct parts, i.e., the outer protective cover called the husk or hull, which is not suitable for human consumption, and the edible portion of the grain called the rice caryopsis or fruit, which comprises the brown rice (Figure 1.1). Brown rice consists of the bran, which comprises the outer layers of the caryopsis along with the embryo, and the endosperm. However, in rice milling, the bran, and the inner layer of the grain (subaleurone) plus a small part of the starchy endosperm, called the polish, are not separated and therefore they are named as bran. The removal of the polish yields common white rice. The hull constitutes about 18-20% of the grain weight and its function is related to protection against insect infestation and rapid changes in moisture content of the grain. The bran portion of the rice accounts for 5-8% of the brown rice

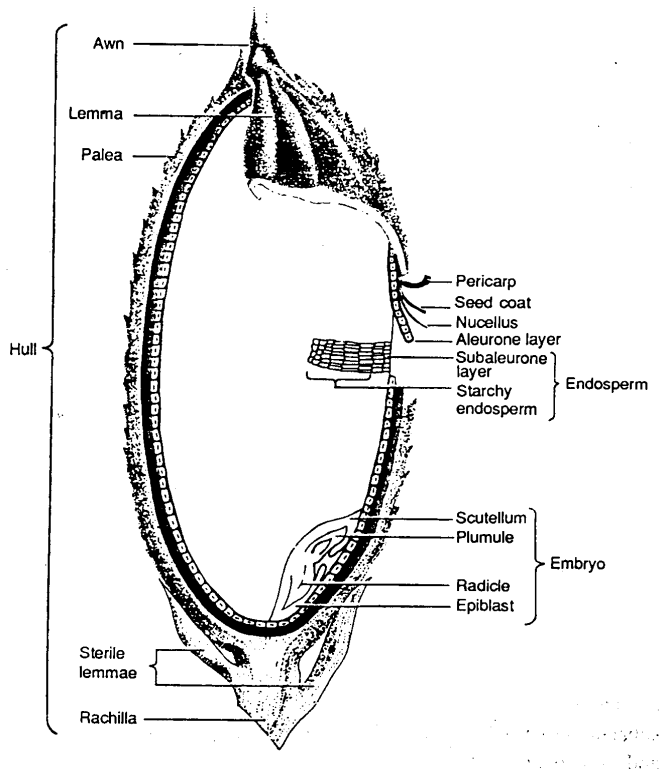

Figure 1.1. Rice grain structure (Juliano, 1985b)

weight and it is the most nutritious part of the caryopsis, since it is rich in protein, phenolics, and lipid bodies. The starchy endosperm, which comprises about 89-94% of the brown rice, is rich in starch granules and contains some protein bodies (6.3-7.1% of total crude protein) but almost no lipid bodies (Juliano and Bechtel, 1985).

1.2.3 Proximate Analysis of Rice Grain

Figure 1.2 shows the rice grain proximate analysis on a dry weight basis. Among all the milling fractions of the rice grain, the bran contains the highest protein content and the hull contains the lowest. Milling of the hull and the bran yields loss of fat, protein,

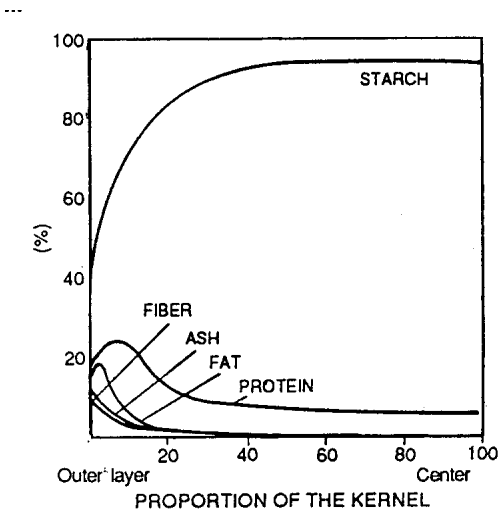

Figure 1.2. Proximate analysis of the rice grain (Barber, 1972).

fiber and ash. However, the carbohydrate content, mainly starch, increases, since it is higher in the endosperm and comprises about 90% of the dry matter in the milled rice. Fiber has its highest content in the hull and the bran, as well as minerals (ash).

1.2.4 By-Products of Rice and their Utilization

During milling of rice several nutrients are lost and a considerable amount of rice grain is discarded. Overall about 30% of the rice grain is lost. These by-products are used for several purposes, as shown in Table 1.1.

1.2.5 Recent Trends in the Use of Rice Phytochemicals

Recently, more and more uses of rice are being seen, its use as a food ingredient are expanding, and its functional and health benefits are being promoted (Pszczola, 2001). The Rockefeller Foundation's International Programme on Rice Biotechnology ensures that new techniques for crop genetic improvement are developed, such as the

Table 1.1. By-products of rice processing and their utilization (Bandyopadhyay and Roy, 1992).

By products	Content of the grain	Uses
Husk or Hull	20% by weight (approx.)	Fuel, white silica or silicon (solar grade); cement and silicates; chemicals and charcoal by dry distillation; furfural, cellulose and xylose; panel board.
Bran	5% by weight (approx.)	Bran oil, both edible and non-edible grades; wax; extracted bran as supplement of protein food or animal feed.
Brokens	-	Rice flour for preparation of baby food or various snack foods.
Germ	2.5-3.0% of brown rice	Baby food supplement.

development of genetically modified crops to improve insect resistance to virus and other insect pests and diseases or environmental stress, which will lead to a stable and high yield-crop (Juliano, 1993). Other new trends in rice development include (1) the development of rice-based ingredients that can help reduce the oil content of foods (Shih and Daigle, 1999) (2) the development of genetically modified rice containing enhanced levels of beta-carotene, called Golden Rice, for which the aim is to reduce the incidence of vitamin A deficiency in Asia, (3) the development of crisp rice product suitable for confectionery products, with clean flavor, such as granola, ice cream toppings, cookies and nutrient bars, (4) the development of a rice cereal with selected flavors infused

directly into the grain, (5) the development of a shelf-stable rice product which does not require cooking, (6) the development of pre-cooked brown rice, which is slightly more nutritious than white rice and aromatic rice, and (7) the formation of newly defined business units focusing on rice that launch rice products with several applications (Pszczola, 2001). Rice bran has been used to develop rice bran-based beverages containing naturally occurring components in bran such as potassium, protein, oil, vitamins and tocotrienols, as well as "rice extracts" which retain the functional and nutritional components of rice bran for use in bread pasta and snacks, doughs and other bakery items. Rice bran fiber and antioxidants have been studied as bioactive compounds for preventing chronic diseases such as cancer hyperlipidemia, fatty liver, hypercalciuria, kidney stones, heart disease, reduction of plasma cholesterol, and antioxidant properties (Pszczola, 2001; Jariwalla, 2001; Deckere and Korver, 1996).

1.3 Rationale of Study

Rice utilization, as a functional ingredient has not been thoroughly investigated. The present work was intended to recover and purify rice cystatin, or oryzacystatin I, a cysteine proteinase inhibitor, whose benefits against chronic diseases have been and are currently being investigated.

The investigation of such compounds has the potential to provide scientific evidence to promote rice consumption and rice fractionation into value-added bioactive compounds, which would lead to an expansion of the rice market.

Oryzacystatin I was recovered and purified from rice bran, where the highest content in protein occurs. Secondly, and as a first approach to the utilization of rice

cystatin as a nutraceutical for eventual use in functional foods, oryzacystatin I oral bioavailability needs to be understood. Therefore, this study also aimed at investigating oryzacystatin I bioavailability in the human body.

CHAPTER 2 LITERATURE REVIEW

2.1 Cysteine Proteinases

2.1.1 Cysteine Proteinases in Health and Diseases

Cysteine proteinases, or thiol proteinases, are small proteins of 23-24 kDa, present in several biological fluids in plants, bacteria, animals and humans. Cysteine proteinases contain two catalytic residues, namely Cys-25 and His-159 (papain numbering). Their pH of optimum activity is between 5 and 6.5, i.e. in mildly acidic and reducing conditions (Henskens et al., 1996). However, there are reports of hydrolytic activity at neutral pH (Buttle et al., 1988). Bacterial cysteine proteinases catalyze the penetration of normal tissues by the bacteria (Henskens et al., 1996) and are equally involved in food digestion. Viral cysteine proteinases, originated from picornaviruses (poliovirus and rhinovirus type I), play a role in the proteolytic cleavage of precursor proteins for virus replication and in the production of new viral particles (Kay and Dunn, 1990; Korant et al., 1986; 1985). For example, HIV-I needs proteolytic processing by cysteine proteinases to regulate the expression of viral proteins. Protozoal cysteine proteinases are involved in the host invasion and in the metabolism of host proteins. They are also involved in the degradation of host immune molecules or in its use for intracellular replication. Mammalian cysteine proteinases are found in lysosomes. The most important mammalian cysteine proteinases are cathepsins B, H, L, and S (Barrett et al., 1988; Barrett and Kirshke, 1981). Cathepsins B, H, L, and S have common ancestors and are related to papain. Lysosomal cysteine proteinases play an important role in intracellular protein turnover (Kominami et al., 1991). They are equally involved in muscle protein turnover

and cleavage of numerous precursor proteins. Also, like collagenases, they are capable of degrading type I collagen (Henskens et al., 1996).

The action of human cysteine proteinases can lead to irreversible damage if, under pathological conditions, lysosomal enzymes are secreted or released by autolysis (Henskens et al., 1996). Alzheimer disease, inflammation, tumor growth and metastasis are typical examples of pathological processes that are the result of imbalance between enzymes and their physiological inhibitors (Blankenvoorde et al., 2000).

2.2 Cystatins

2.2.1 Definitions, Sources, and Mechanism of Action

Cystatins are the natural inhibitors of cysteine proteinases (Henskens et al., 1996). Conventionally, they are divided in three major families according to their amino acid sequence (Barrett et al., 1986a) as shown in Table 2.1. However, this classification is based only on animal cystatins, because many studies have been conducted to clarify the physiological and clinical significance of cystatins in animal tissues and products. Cystatins have received much attention in the last two decades due to their potential in regulating the function of cysteine proteinases (Henskens et al., 1996). The major characteristics of mammalian cystatins are summarized in Table 2.1.

During the last decade, a fourth group belonging to the cystatin superfamily has emerged, that is, the plant cystatins. The first plant cystatin was found by Abe and Arai (1985) in rice seeds and is called oryzacystatin. Several other plant cystatins were discovered in soy, corn, wheat, potato, ragweed, cowpea, avocado, and papaya. Homology searches show that plant cystatins, except for potato cystatin, resemble family

Table 2.1. Major characteristics of mammalian cystatins.

Characteristics	Family 1	Family 2	Family -3
Amino acid residues	About 100	115 – 120	3 cystatin like domains
Molecular weight (kDa)	About 11	13 – 14	High: 88-114 Low: 50-68
Disulfide bonds	0	2	6
Glycosilated	No	No	Yes
Location	Mainly intracellularly	Mainly extracellularly	Intravascularly
Cystatins	Human: A (stefin A), B (stefin B) Rat: cystatin α and β	Human: C, D, S, S1, S2, SN, SA, D Rat: C, S Mouse: C Chicken egg white, bovine colostrums, ox, Drosophila.	Human, rat, bovine: L-kininogen, H-kininogen Rat: T-kininogen Ox: kininogen.

2 cystatins of animal origin, but lack disulfide bonds like family 1 cystatins (Arai and Abe, 2000). Despite the high protein sequence homology, the gene organization of oryzacystatins is markedly different to that seen in animal cystatins. Therefore, it has been suggested that a new cystatin family exists, namely the phytocystatins (Brown and Dziegielewska, 1997).

Most cystatins are reversible, tight binding competitive inhibitors of cysteine proteinases, which form equimolar complexes with their target enzymes (Blankenvoorde et al., 2000). Their general mechanism of action is based on three domains that show highly conserved amino acids sequences. These are important for the inhibitory activity. These domains consist of 10 amino acid residues in the amino terminus, a β-hairpin loop containing the conserved –QVVAG- residues, and a second β-hairpin loop containing the conserved residues Leu102, His 104 in family 1 and Trp104 in family 2 cystatins (Calkins and Sloane, 1995). This wedge penetrates and covers the active site in such a fashion to block the papain or other cysteine proteinase's active site cysteine residue (Blankenvoorde et al., 2000; Calkins and Sloane, 1995). The interactive elements of this complex are represented having chicken egg-white cystatin as model in Figure 2.1 (Turk and Bode, 1991).

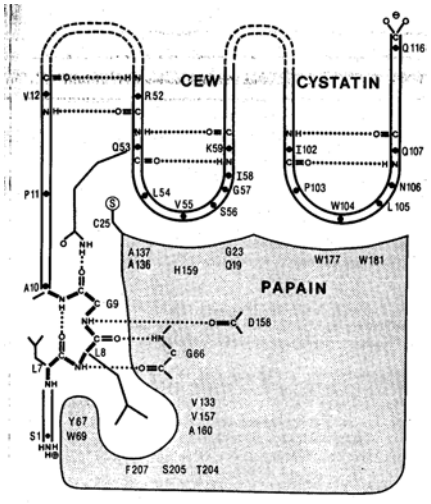

Figure 2.1. Scheme of the proposed "trunk model" for the interaction of chicken egg-white cystatin (cew cystatin) and papain (Turk and Bode, 1991).

2.2.2 Cystatins in Health and Diseases

Alterations to the proteinase inhibitors-cysteine proteinase ratios contribute to the progression of several pathological processes. Cystatins have been shown to play a key role against viruses, bacteria, and parasites, in the control of tumor growth and metastasis, in the protection against tissue destruction, in hereditary cystatin C amyloid angiopathy, in neurological disorders, and as a marker of glomerular filtration rate (reviewed by Blankenvoorde et al., 2000; Henkens et al., 1996).

Many viruses require proteolytic cleavage to become infectious. Cystatins possess activity against a variety of viruses such as poliovirus, rhinovirus, coronavirus, and herpes simplex virus (Aoki et al., 1995; Kondo et al., 1992; Korant et al., 1988; 1986; 1985). Although most likely the antiviral activity of cystatins is due to their proteinase inhibitory properties, the viral target enzymes have not been identified yet (Blankenvoorde et al., 2000).

Cystatins have antibacterial properties as well since they play a role in the inhibition of bacterial cysteine proteinases when penetrating normal tissues. Cystatins inhibited the growth of *Porphyromonas gingivalis* (Blankenvoorde et al., 1997; 1996; Naito et al., 1995; Grenier, 1992;) and *Staphylococcus aureus* (Takahashi et al., 1994).

Cysteine proteinase inhibitors have also been reported as inhibitors of parasite infections such as malaria. In some parasitic infections, the parasite obtains free amino acids for protein synthesis via the action of cysteine proteinases, which intracellularly degrade the host proteins. Inhibition of these proteinases correlates with blocking the protein degradation and killing of cultured parasites (Blankenvoorde et al., 2000). A parasitic cysteine proteinase was inhibited by cystatins, namely that of *Entamoeba histolytica*, which is thought to play an important role in tissue invasion (Luaces and Barret, 1988).

Cysteine proteinases of cancer cells may facilitate the growth of the tumor due to their ability to degrade stromal tissues and basement membranes. Cysteine proteinases have been implicated in cancer malignancy by activating proproteinases like precursors of metalloproteinases (Berquin and Sloane, 1996; Schmitt et al., 1992). Cysteine proteinases can interfere with chemotherapy due to the inactivation of antitumor drugs such as the case of bleomycin (Blankenvoorde et al., 2000).

The onset/progression of malignant tumor cells is due to an imbalance between cysteine proteinases and their inhibitors (Calkins and Sloane, 1995). However, contradictory data have been shown on cystatin activity in malignant tumors. Cystatin activity has been shown to be higher, similar, or lower compared to the activity found in normal tissues (Blankenvoorde et al., 2000; Knoch et al., 1994; Lah et al., 1992, Lah et al., 1989). Others consider that cystatins have an opposite effect in the process of malignancy. They consider that excess of cystatin C could inhibit the proteolytic attack of cathepsins on the cancer cell by suppressing the host inflammatory response and in this way enhancing the oncogenicity of the cell (Henskens et al., 1996). Cystatin C inhibits motility and *in vitro* invasiveness of cancer cells, supporting the hypothesis that cystatins play a role in the maintenance of cell differentiation (Blankenvoorde et al., 2000).

Schelp and Pongpaew (1988) and Troll et al. (1984) suggested that proteinase inhibitors present in cereals like rice and maze can prevent certain types of cancer. Bjornland et al. (1996), ShojiKasai et al. (1988), and Saito et al. (1980) have reported antitumor activity of cysteine proteinase inhibitors, E-64 and leupeptin, by selective reduction of the growth of transformed cells and reduction of the occurrence of cancer in animal models. Other proteolytic enzymes may also play a role in tumor growth. Cathepsin D inactivates cystatins. Inhibitors of cathepsin D

may not only prevent tumor growth (Ren and Sloane, 1996), but also prevent the inactivation of cystatins by cathepsin D (Lenarcic et al., 1988), resulting in an accumulative inhibition of tumor growth (Blankenvoorde et al, 2000).

Cysteine proteinases activate proinflammatory mediators, and catalyze tissue degradation. Periodontitis and rheumatoid arthritis are inflammatory diseases catalyzed by cysteine proteinases (Blankenvoorde et al., 2000). Smoking is associated with lower cystatin activity during gingival inflammation (Lie et al., 2001). Cathepsins B and L are enzymes associated with the onset of rheumatoid arthritis and higher levels of the enzymes are found in synovial tissues and fluids of arthritis patients (Buttle et al., 1995). Therefore, cathepsin B seems to be a good target for pharmacological intervention. Cysteine proteinase inhibitors anti-inflammatory and anti-rheumatic drugs successfully reduce cysteine proteinases that catalyze tissue destruction in rheumatoid arthritis (Blankenvoorde et al., 2000; Yamamoto et al., 1984; Kruze et al., 1976).

Cystatin C as an indicator of kidney function has been suggested. Low molecular weight proteins are eliminated from the circulation by glomerular filtration followed by reabsorption and catabolism. In healthy individuals the blood cystatin C level is constant. Serum levels of cystatin C are much more constant than creatinine levels, the best-known marker of glomerular filtration rate. The plasma level of cystatin C only rises as renal function fails (Henskens et al., 1996). Newman at al. (1995) reported an assay using cystatin C that showed to be more sensitive as a screening test for early renal damage than creatinine.

2.2.3 Potential Food and Pharmaceutical Applications of Cystatins

Proteinases in muscle from various fish species cause severe and rapid textural degradation during cooking (Benjakul et al., 2001). Naturally occurring proteinase inhibitors

have the ability to prevent fish tissue degradation associated with the proteinases. Successful applications have included the use of beef plasma, whey protein isolates, egg white, potato extract, and lactoalbumin to prevent fish tissue softening (Izquierdo-Pulido et al., 1994). Recently, cystatins were reported for eventual use as inhibitors of disintegration of fish such as minced fish (surimi). Examples are carp ovarian cystatin (Tzeng et al., 2001) and chicken cystatin (Jiang et al., 2002). Those expressed in *E. coli* in a controlled manner were suitable for industrial use (Jiang et al., 2002; Tzeng et al., 2001). The authors report its possible use as inhibitors of surimi gel softening. Benjakul et al. (2001) and Izquierdo-Pulido et al. (1994) have reported applications of rice cystatins versus proteases in Arrowtooth flounder and Pacific whiting, respectively, the two fish species used in surimi manufacturing.

According to Section 2.2.2, the potential exists to utilize cystatins in food and pharmaceutical formulations as inhibitors of enzymes associated with the onset and/or progression of a wide range of pathological processes. Poliomyelitis caused by poliovirus may be prevented with cystatins. Other pathological processes such as inflammations, infections, osteoporosis, and cancer may also be prevented by cystatins. Cystatins may also find application in the prevention gingivitis and periodontitis (Blankenvoorde et al., 2000).

2.3 Oryzacystatins

2.3.1 Characterization of Oryzacystatins I and II

Rice grain contains cysteine proteinase inhibitors, termed oryzacystatins I and II (OCI and II). OCI is a polypeptide with a molecular size of about 11.5 kDa (determined by SDS-PAGE and amino acid analysis) and 12 kDa (determined by gel filtration using Sephadex G-75) (Abe and Arai, 1985). OCI occurs in a number of rice cultivars at an average of 2-3 mg/Kg of seed (Arai and Abe, 2000). OCI is very heat stable, (Arai and Abe, 2000), can withstand

temperatures as high as 100°C for 30 minutes, but loses up to 15% of its activity at temperatures of 110°C or higher. OCI is stable below pH 9.0, but its activity is decreased at pH 10 or higher (Abe et al., 1987). The amino acid sequence of OCI has a significant similarity to those of representative cystatins of animal origin, which led to the suggestion that this compound belongs to the cystatin superfamily (Arai and Abe, 2000). OCI bears no disulfide bonds, therefore it could be classified as family 1. However, in terms of the sequence homology, OCI resembles more closely to family 2 cystatins (Abe et al., 1987). OCI has 102 amino acids, and no half-cystine residues. The predominant amino acids include Asp, Glu, Val, Ala, and Lys (Abe et al., 1987). OCI, like animal cystatins, has the conserved central pentapeptide Gln-Val-Val-Ala-Gly as a probable target enzyme-binding site, but lacks disulfide bonds. OCI inhibits papain in a stoichiometric manner, with a K_i value of 10^{-8}M. However, OCI inhibits cathepsin H with a K_i value of 10^{-6} (Arai and Abe, 2000). OCI also inhibits ficin, but to a lesser extent. However, it failed to inhibit bromelain by only inhibiting up to 4% or less (Abe et al., 1987). OCI does not inhibit serine proteinases (trypsin, chymotrypsin, and subtilisin), aspartic proteinases, or carboxyl proteinases (Abe et al., 1987).

OCII shows 59% identity with OCI in terms of nucleotide sequences. This OC has a molecular size of 12 kDa, as shown by SDS-PAGE (Kondo et al., 1990). OCII is composed of 107 amino acid residues. Like OCI, it does not contain disulfide bonds, and can be classified as belonging to family 1 of the cystatin superfamily. However, with respect to amino acid sequence, OCI, like OCII are more closely related to family 2 cystatins. Again, the commonly conserved sequence Gln-Val-Val-Ala-Gly in the central part of the molecule of most cystatin superfamily members including OCI is also present in OCII. OCII gives a K_i value of 10^{-6} against papain and a K_i value as small as 10^{-8} against cathepsin H (Arai and Abe, 2000).

OCI and OCII inhibit papain and cathepsin H non-covalently (Arai and Abe, 2000). Neither OCI nor OCII showed any effective inhibition against cathepsin B or L (Kondo et al., 1990).

2.3.2 Endogenous and Exogenous Cysteine Proteinases as Targets of Oryzacystatins

OCI and OCII occur in ripened rice seeds and possibly play a role in regulating the actions of cysteine proteinases present in rice (Abe et al., 1991). Three molecular species of cysteine proteinases present in rice, named oryzains α, β, and γ (OZ-α, OZ-β, and OZ-γ) are the probable endogenous target enzymes of OCI and OCII, since their proteolytic activities are well regulated by these cystatins *in vitro*. OZ-β is involved in proteolysis of seed storage proteins, OZ-α is involved in proteolysis of some functional proteins in the aleurone, and OZ-γ is involved in intracellular protein turnover (Arai et al., 1998). OZ-α and OZ-β are very homologous to papain, whereas OZ-γ shows a very high homology to cathepsin H. Therefore, it is highly probable that in rice seeds OZ-β as well as OZ-α functions as proteinases of the papain-type and OZ-γ functions as a proteinase of the cathepsin H-type. The function of these three proteinases of the two types may contribute to either or both of the processing for maturation of storage proteins such as glutenin during ripening of rice seeds and the proteolysis for degradation of the storage proteins during germination (Abe et al., 1991). Considering the fact that OCI and OCII inhibit papain and cathepsin H, respectively, it is likely that OZ-α and OZ-β as papain-type enzymes and OZ-γ as a cathepsin H-type enzyme are the natural target enzymes of OCI and OCII, respectively (Abe et al., 1991).

There are a variety of human viruses including picornaviruses that enter the intestine *per se*. These viruses in particular, when entering animal cells, synthesize a high-molecular weight polyprotein, which is subsequently divided into a number of viral components. The cleavage of

this polyprotein is carried out by virus-encoded proteinases, among them a cysteine proteinase. Therefore, the inhibition of this viral cysteine proteinase by cystatin would lead to stopping the proliferation of virus particles in the infected cells. Arai et al. (1995) used poliovirus as an example of picornavirus. The virus yields in the presence each OCI and OCII remained much lower than that obtained in its absence. OCI also showed a strong inhibitory effect on the proliferation of herpes simplex virus. Though nothing is known experimentally concerning the mechanism involved in these processes, it is speculated that OCI molecules enter the infected cells by internalization to inhibit the molecular event in which the polyprotein is processed into functional products (Arai et al., 1998). These results suggest that the use of a cystatin as a proteinaceous substance is important in obtaining an antiviral effect. Also, it is inferred that phytocystatins like OCI and OCII can be seen as useful factors for molecular breeding of virus resistant crop cultivars, as well as of functional food materials with an antiviral effect (Arai et al., 1995).

Plants use a variety of proteinase inhibitors as bioprotectants versus pests and insects. Plant proteinase inhibitors inactivate proteinases in the digestive tract of insects or pests. Despite this, most crops are still susceptible to damage by specific insect pests. Parasites from cowpea and soybeans were fed with diets containing OCI and II. Results showed that OCI retarded growth, or even killed those insects at higher concentrations (Kuroda et al., 1996). Another pest from rice seeds was tested, namely *Sitophilus zeamais*. Both OCI and II retarded the growth of the rice pest and even killed it at larval stage (Arai et al., 1998). This suggests that cystatins, which do not interfere with protein digestion in humans, may target specific cysteine proteinase inhibitors of insect pests (Arai and Abe, 2000).

CHAPTER 3 PERMEABILITY OF ORYZACYSTATIN I ACROSS CACO-2 CELLS

3.1 Introduction

Bioavailability studies of oryzacystatin I (OCI) are useful because they provide an insight of the protein pathway in the human body. To this effect, it is necessary to study the changes OCI undergoes when ingested, that is, the effect that the proteinases present in the human gastrointestinal tract show towards this compound. Also, it is necessary to clarify if the protein or its derived peptides are in actual fact absorbed by the human body in the small intestine. Therefore, conditions mimicking the human gastrointestinal tract including the small intestine need to be studied.

Small intestinal epithelial cells are the primary sites of absorption of nutrients. Absorption through the intestine requires that such nutrients cross two membranes, namely the epithelial cells from the lumen across the brush-border membranes followed by the transfer to the blood across the basolateral membranes (Terada et al., 1999). Net transepithelial flux is composed of transcellular and paracellular simple diffusion in both directions, activity of one or more transport systems, intracellular accumulation and degradation, efflux across the luminal cell membrane back into the apical compartment, and transport across the basolateral membrane (Bretschneider et al., 1999). The most discussed carrier present in the intestinal epithelia responsible for peptide and peptide-like delivery is the peptide transporter PEPT1. PEPT1, expressed and localized at the brush-border membranes is driven by a transmembrane H^+ gradient and catalyzes the cotransport of its substrates like di- and tri-peptides with H^+. The intestinal peptide transporter is an important determinant of the bioavailability of peptides and peptide-like drugs (Bretschneider et al., 1999). A facilitative, not H^+-coupled peptide transport

system is localized in the basolateral membrane and is responsible for the transport of small peptides and peptide-like drugs across the basolateral membrane (Terada et al., 1999).

Caco-2 cell monolayers are an excellent model of intestinal transport. Caco-2 cells are derived from human colon adenocarcinoma and very much mimic the morphology and features of the intestinal epithelia. They can be grown in semipermeable filters and spontaneously differentiate in culture to form a confluent monolayer. This monolayer resembles the small intestinal epithelium, both structurally and functionally. In recent years, Caco-2 cells have become screening tool for intestinal absorption of drugs, prediction of transport routes, and rates of flux (Gabor et al., 2002; Adebowale et al., 2000; Bretschneider et al., 1999). Other compounds whose bioavailability has been studied using Caco-2 cells include amino acids, steroid hormones, trace elements, and antibiotics (Bretschneider et al., 1999; Finley et al., 1995). PEPT1 and the basolateral carrier are also expressed in the human intestinal cell line Caco-2 (Terada et al., 1999).

The present study focused on the transport of OCI or its chymotryptic peptides across Caco-2 cells, the system chosen to mimic intestinal absorption, in order to determine the bioavailability of OCI.

3.2 Materials and Methods

OCI was purified from rice bran using a modification of the process described by Abe et al. (1987). The purification process is outlined in Figure 3.1.

3.2.1 Preparation of Rice Bran Homogenate

One kilogram of defatted rice bran was homogenized with 3 L of 25 mM sodium phosphate buffer containing 0.15 M sodium chloride, pH 7.0. The mixture was shaken in an Environ-Shaker (Biomedevice Engineering, San Francisco, CA) at room temperature for 3 hours.

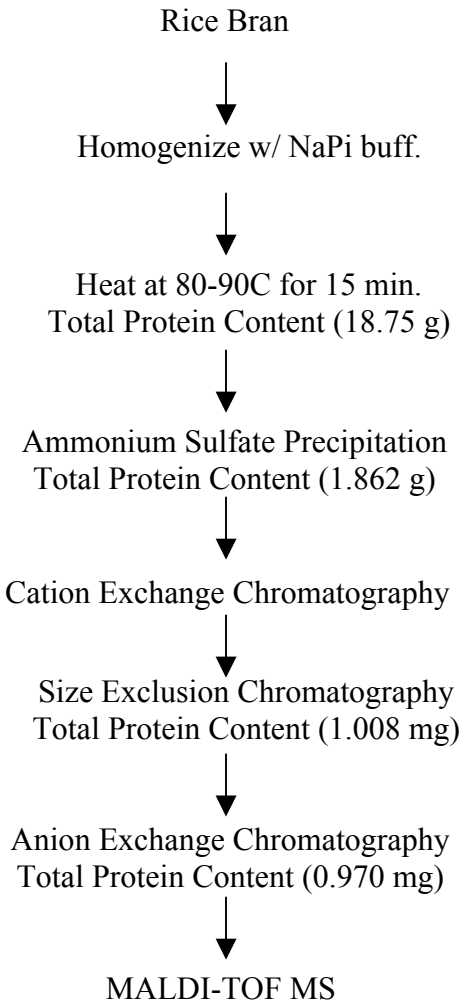

Figure 3.1. Purification procedure of OCI.

The homogenate was centrifuged at 8,000 × *g* for 20 min at 4˚C to remove residues. A volume of 1.9 L was obtained after centrifugation. The supernatant from centrifugation was immediately heated in a water bath with an average temperature of 94˚C. The time required for the sample to reach 80˚C was 18 min. The sample was kept in the water bath for 15 min and the final temperature was 90˚C. This process aimed at precipitating heat labile proteins, keeping in solution heat-stable proteins. After cooling down, the precipitated material was removed by

centrifugation at 8,000 × *g* for 20 min at 4˚C and the supernatant collected. A volume of 1.88 L of homogenate was obtained at this point.

3.2.2 Ammonium Sulfate Precipitation

Solid ammonium sulfate was added to the total volume of homogenate to obtain a precipitate formed between 0 and 20% saturation, 20 and 50% saturation, and 50 to 80% saturation. The precipitates obtained as well as 10 ml of the solution that did not precipitate (>80%) were dialyzed overnight against 50 mM sodium acetate buffer pH 4.8 (buffer A). The dialyzed samples were checked for papain inhibitory activity by the method of Abrahamson (1994), as described below. The sample obtained between 50-80% saturation, which showed the highest papain inhibitory activity, was further dialyzed overnight against buffer A.

3.2.3 Cation Exchange Chromatography

The dialyzate obtained from the 50-80% ammonium sulfate saturation fraction was centrifuged at 13,000 rpm (bench top centrifuge) for 1 min at room temperature to remove non-dissolvable material and filtered through a 0.45 μm filter. The filtrate was loaded on a HighPrep 16/10 CM column (Amersham Pharmacia Biotech, Piscataway, NJ). The column was equilibrated with 20 ml of buffer A, the sample (70 ml) was injected and the column was washed with 50 ml of buffer A. The column was eluted by a linear gradient of 0-0.5 M sodium chloride at a flow rate of 4.0 ml/min using an Äkta Prime purification unit (Amersham Pharmacia Biotech, Piscataway, NJ). Samples of 5.1 ml were collected and a total volume of 400 ml was collected. The absorbance at 280 nm for each fraction was read in a Spectramax Plus equipment (Molecular Devices, Sunnyvale, CA). The papain inhibitory activity was checked. Fractions with papain inhibitory activity were individually freeze-dried. The non-bound fraction obtained after injection (ca. 90 ml) was ultrafiltered through a 1 kDa molecular weight cut-off (MWCO)

membrane in a Normal Flow Filtration module (Millipore, Bedford, MA) until the volume was concentrated to ca. 15 ml (6-fold concentration).

3.2.4 Size Exclusion Chromatography

The lyophilized powder from the fraction with the highest papain inhibitory activity was dissolved in 520 µl of 25 mM phosphate buffer pH 7.0 containing 0.15 M sodium chloride (buffer B) and centrifuged at 13,000 rpm (bench top centrifuge) for 1 min at room temperature to remove indissolvable material. The supernatant was loaded onto a HiPrep Sephacryl 16/60 S-200 HR column (Amersham Pharmacia Biotech, Piscataway, NJ) after the column had been equilibrated with 240 ml of buffer B. The column was eluted with buffer B using an Äkta Prime (Amersham Pharmacia Biotech, Piscataway, NJ) purification unit at a flow rate of 0.5 ml/min Samples of 2 ml were collected throughout the elution. The absorbance of each 2 ml fraction was read at 280 nm and the papain inhibitory activity of the fractions was measured using the method of Abrahamson (1994), as described below. The fractions with papain inhibitory activity were individually freeze-dried.

3.2.5 Anion Exchange Chromatography

The fraction powder that showed the highest papain inhibitory activity from size exclusion chromatography was mixed with 400 µl of filtered water and mechanically dissolved. The mixture was centrifuged at 13,000 rpm (bench top centrifuge) for 1 min to remove non-dissolvable material and the supernatant (290 µl) collected and kept at 4˚C until further use. This fraction was dialyzed overnight at 4˚C against 25 mM sodium phosphate buffer pH 7.5 (buffer C). The final volume achieved after dialysis was 1500 µl. The volume was increased to 2 ml and loaded onto a HiPrep 16/10 DEAE (Amersham Pharmacia Biotech, Piscataway, NJ) column after the column had been equilibrated with 60 ml of buffer C. The column was washed with 50 ml of

buffer C. The column was eluted with a linear gradient of 0-0.5 M sodium chloride using an Äkta Prime (Amersham Pharmacia Biotech, Piscataway, NJ) purification unit at a flow rate of 4.0 ml/min. Samples of 6.3 ml were collected and a total volume of 400 ml was collected. The absorbance of the eluted fractions was read at 280 nm and the papain inhibitory activity was measured.

3.2.6 MALDI of OCI

MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionisation-Time Of Flight Mass Spectrometry) is a molecular ionization technique discovered simultaneously and independently by Tanaka et al. (1988) in Japan and Karas and Hillenkamp (1988) in Germany. The principle of MALDI is that a laser beam supplies energy to the molecule of interest, which has been mixed with a matrix. The laser causes chemical ionization of the matrix and the analyte. The analyte is protonated and the ions produced are accelerated in a tunnel by a high voltage (about 20,000 V) toward an ion detector system. The mass of the analyte is calculated by the time of flight. MALDI allows obtaining the molecular weight measurements from picomole amounts of biopolymers. Accurate molecular weights can be obtained quickly and easily for materials from small peptides and oligosacharides to intact proteins up to 250,000 Daltons.

To verify the molecular weight of the purified protein, the fraction with most inhibitory activity obtained after anion exchange was thoroughly dialyzed against filtered water and diluted to a concentration of 150 and 4 pmol/µl (in water), respectively, assuming a molecular weight of 12,000 for OCI. The samples were analyzed by a Bruker Triflex II MALDI spectrometer. OCI was mixed at 150 and 4 picomoles with saturated solution of sinapinic acid as the matrix. Matrix and samples were deposited on the MALDI probe and allowed to air-dry. Spectra were recorded

using a Bruker Triflex II operating at 20,000 V with the nitrogen laser focused at 337 nm. At least fifty shots were averaged to obtain a decent spectrum of OCI.

3.2.7 Hydrolysis of OCI

Purified OCI was hydrolyzed with chymotrypsin at 1:100 (enzyme: protein ratio) in 20 mM sodium phosphate buffer pH 7.4 at 37˚C for 2.5 hours. After the incubation, the mixtures were heated at 90-95ºC for 15 min to inactivate and precipitate chymotrypsin. OCI and their chymotryptic peptides remained in the solution, since this protein is very heat stable. SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) with silver staining was run, as described below, to check for peptides derived from OCI hydrolysis.

3.2.8 Caco-2 Cells Permeability Study

The flux of OCI and the chymotryptic derived peptides from the apical to the basolateral side was evaluated following the protocol outlined by *In Vitro* Technologies, Inc. (2001). Caco-2 cells and Transwell insert filters (diameter 24 mm, pore size 0.4 μm) were obtained from the Cell and Organ Culture facility at School of Veterinary Medicine, Louisiana State University. Caco-2 cells after reaching confluency on semi-permeable Transwell filters were used. The dosing solutions were loaded on the apical side of the Transwell filters, and the permeate collected on the basolateral side, as shown in Figure 3.2.

Prior to the experiment, the seeded Caco-2 cells were washed both in the apical and in the basolateral side with Dulbecco's phosphate buffer saline (DPBS). The purified OCI and the chymotryptic derived peptides were tested for this experiment. DPBS was used as a control for the purified OCI. The control used for the chymotryptic-derived peptides consisted of chymotrypsin processed under the same conditions as for the hydrolysis, but without OCI. One hundred and fifty μl of each solution, including the two controls (named dosing solutions) were

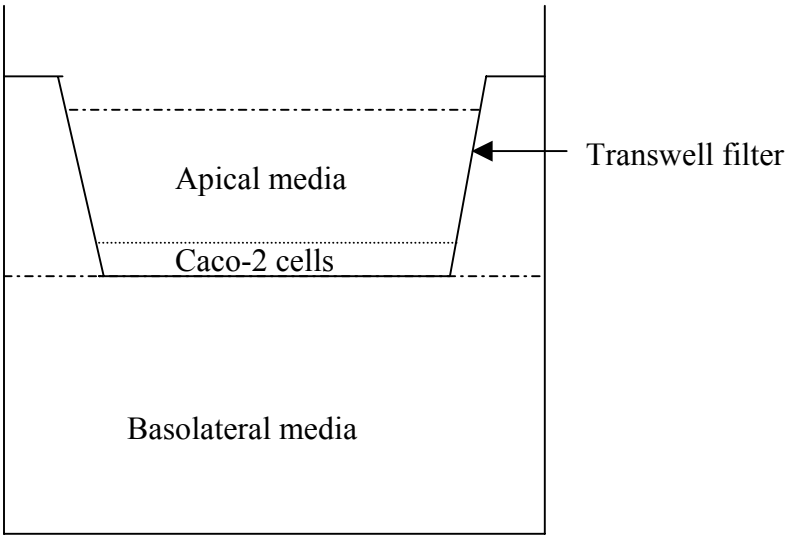

Figure 3.2. Scheme of the Caco-2 cells permeability study.

added to the apical side of the individual Transwells. Two hundred µl of DPBS was added to the basolateral side of each Transwell. The plate was placed on an orbital shaker and kept at 37˚C inside a 5% CO_2 humidity incubator. All samples were run in triplicate. The plate was incubated for 3 hours and removed from the incubator after that time. The remaining apical media and the basolateral media collected after the experiment were transferred to individual eppendorf microcentrifuge tubes and kept at 4˚C for further analysis. The amount of OCI was quantified in each dosing solution, in the remaining apical media, and in the media on the basolateral side of each Transwell based on papain inhibitory activity assay. Molecular size of OCI peptides collected in the apical and basolateral side of the Transwells after the incubation was determined by MALDI-MS according to the procedure described in Section 3.2.6.

3.2.9 SDS-PAGE

Electrophoresis of OCI fractions from ammonium sulfate precipitation, ion exchange chromatography, size exclusion chromatography, and chymotrypsin hydrolysis was carried on 4-

12% BisTris gel under denaturating conditions in the presence of reducing agent. Electrophoresis separation was accomplished in a Mini VE separation unit (Amersham Pharmacia Biotech, Piscataway, NJ) at 180V for an average of 1 hour. Gels for ammonium sulfate precipitation, cation exchange, and size exclusion chromatography were stained with Coommassie Blue. Gels for anion exchange chromatography and OCI peptides were silver stained following the manufacturer instructions.

3.2.10 Papain Inhibitory Activity

Papain inhibitory activity of the ammonium sulfate precipitation fractions, ion exchange and size exclusion chromatography fractions, chymotryptic peptides, and apical and basolateral fractions of the Caco-2 cells experiment was determined by the method of Abrahamson (1994), with modifications. This method is based on the principle that 1 mole of OC inhibits 1 mole of papain (Abe et al., 1987). In brief, 125 µl of 500 mM sodium phosphate buffer pH 6.5 were mixed with 50 µl of 5 µM papain and 50 µl of sample. The mixture was vortexed and the samples were incubated at room temperature for 15 minutes. The reaction was started by addition of 15 µl of 100 mM benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA) in dimethyl sulfoxide (DMSO) and incubated at 37ºC for 1 hour. The reaction was stopped by addition of 50 µl of 30% acetic acid. After centrifuging at 13,000 rpm (bench top centrifuge) for 5 minutes, absorbance was read in an ELISA plate reader Spectramax Plus at 410 nm (Molecular Devices, Sunnyvale, CA). The blanks consisted of complete mixtures incubated for 0 hours where the stopping reagent was added prior to the BAPNA substrate. The positive control consisted of adding water instead of OCI sample. Papain inhibitory activity (%) was calculated as [Abs(control)-Abs(sample)]/Abs (control)*100. Specific activity was defined as 1 unit per µg of protein. One unit was defined as 1% of papain inhibitory activity (for 250 nmol of papain).

3.2.11 Total Protein Content

Total protein content of the ammonium sulfate precipitation fractions, anion exchange and size exclusion chromatography fractions, solution of chymotrypsin used in the hydrolysis process, and the apical and basolateral media collected after the permeability study was determined. The BCA Protein Assay Kit (Pierce, Rockford IL) was used and procedure was done according to the manufacturer instructions. Since the assay only performs in basic conditions, a 200 mM sodium phosphate buffer pH 8 was used to dilute the samples.

3.3 Results and Discussion

3.3.1. Purification of OCI

The procedure used for purification of OCI and the data obtained throughout the purification process are summarized in Table 3.1. Overall, OCI was successfully purified, giving a single protein band on SDS-PAGE (Figure 3.10) and a single peak at 11.5 kDa by MALDI-TOF MS (Figure 3.11). Almost 1 mg of OC was recovered from 1 kg of rice bran. More than 14-fold increase in specific activity was achieved throughout the purification process.

Table 3.1. Purification of rice cystatin I.

Purification procedure	Total protein (mg)	Specific activity (units/μg)
Heat-treated homogenate	18,754	0.1342
50-80% $(NH_4)_2SO_4$ precipitation	1,682	0.05420
Cation exchange chromatography	- *	- *
Size exclusion chromatography	1.008	2.946
Anion exchange chromatography	0.970	1.924

* Not determined.

3.3.1.1 Ammonium Sulfate Precipitation

The first step carried out to purify OCI was the fractionation of the heat-treated rice bran proteins using ammonium sulfate precipitation. Table 3.2 shows the data obtained throughout the ammonium sulfate precipitation procedure.

Table 3.2. Ammonium sulfate precipitation of the rice bran homogenate.

Precipitation Range (%)	Volume (ml)	Total Protein Content (g)	Total Papain Inhibitory Activity (units)
0-20	41	0.1760	22.28
20-50	152	2.314	94.65
50-80	72	1.862	50.45
>80	1880	0.8374	811.4

As seen on Table 3.2, the precipitates obtained between 0 and 20% saturation with ammonium sulfate and the non-precipitated fraction (>80%) showed the lowest total protein content, which was an indication that OCI was not present in these fractions. However, the fraction that did not precipitate up to 80% saturation with ammonium sulfate yielded a high total papain inhibitory activity. The high activity may be related with other compounds than proteins present in the non-precipitated rice bran fraction. The precipitates obtained between 20 and 50% and between 50 and 80% saturation with respect to ammonium sulfate showed the highest total protein content among the precipitated fractions (Table 3.2). The fraction obtained between 20 and 50% saturation yielded higher total papain inhibitory activity than the 50-80% saturation fraction. However, in the first fraction a large volume of precipitated material was obtained

(Table 3.2), which contained a large amount of unwanted material. OCI was believed to be present in the fraction that precipitated in the 50-80% saturation range, since that fraction also yielded a high total papain inhibitory activity. However, a decrease in specific papain inhibitory activity between the heat-treated homogenate and the 50-80% ammonium sulfate precipitation step was observed (Table 3.1). Abe et al. (1987), who used a similar approach to recover OCI, used wider precipitation ranges (30-65%) and obtained a five-fold increase in specific activity up to this step. Comparison between the two methods should be done with care, since Abe et al. (1987) used rice seeds as starting material, whereas rice bran was used in the present work. Results show that by using a narrower precipitation range, unwanted proteins and other material were discarded at the 20-50% ammonium sulfate precipitation range, therefore it seems more appropriate to use narrower precipitation ranges. The fact that in the 20-50% fraction, a high amount of protein precipitated (Table 3.2), raised the possibility that some OCI might have precipitated at the 20-50% range. However, the fact that it was possible to eliminate a very high amount of unwanted material overcomes the possible loss of OC, as seen also in the decrease in specific activity up to this step (Table 3.1). Therefore, the precipitation between 50 and 80% seems more appropriate in terms of removal of unwanted compounds and recovery of OCI.

As seen in Figure 3.3, in the precipitate obtained between 50-80% saturation, a large amount of protein was concentrated in the 6-30 kDa range, which is an indication of the presence of OCI (lane 5). The precipitate obtained between 0 and 20% (lane 2) and the solution containing material that did not precipitate up to 80% ammonium sulfate (lane 6) seemed lower in protein content than the fraction precipitated at 50-80%. The fraction that precipitated below 20% and the non-precipitated fraction (>80%) yielded the lowest total protein content (Table 3.2), which correlates well with what was observed by SDS-PAGE. The 50-80% fraction was pooled for

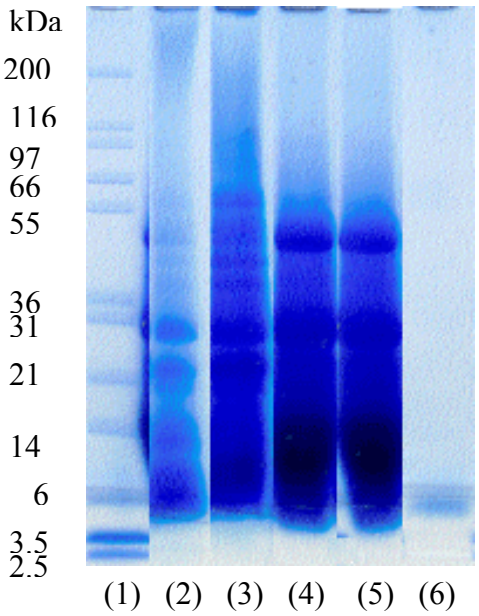

Figure 3.3. SDS-PAGE with Commassie Blue staining of rice protein obtained throughout ammonium sulfate precipitation.
Legend:
(1) molecular weight markers (molecular weight (kDa) is shown on the left-hand side of the SDS-PAGE picture)
(2) fraction precipitated between 0-20%
(3) fraction precipitated between 20-50%
(4) re-centrifuged fraction precipitated between 50-80% (cation exchange injected sample)
(5) fraction precipitated between 50-80%
(6) non precipitated fraction (>80%)

further purification, i.e., for use in ion exchange chromatography. Therefore, the fraction was re-centrifuged to assure total solubility of the fraction to use and thus avoid the aggregation of possible insoluble material in the chromatography column. As seen in Figure 3.3 (lane 4), no protein seemed to have been removed with the centrifugation process, as the location of the bands and the band intensity remained the same. The total protein content found in the fraction after the centrifugation process correlates well with what was seen on SDS-PAGE, since only 7% of protein was lost.

3.3.1.2 Cation Exchange Chromatography

The 50-80% ammonium sulfate precipitation fraction was loaded on a HiPrep 16/10 CM column to separate the group of proteins present in the sample based on their ionic properties.

Figure 3.4 (A) shows the elution profile of the sample loaded on the CM column. A peak was observed eluting at around 107 ml. However, further analysis proved that this peak did not correspond to the OCI elution, as seen on papain inhibitory activity on Figure 3.4 (B). A visual observation of the eluted samples showed that the high absorbance readings seen at 107 ml elution volume were related to the yellowish compound present in the rice bran. The elution profile proved again that until this purification step, the sample contained a large mixture of proteins, since relatively high absorbance readings are found throughout the elution volume. Figure 3.4 (B) shows the papain inhibitory activity on fractions collected throughout the cation exchange elution process. Compounds with papain inhibitory activity eluted in early stages of the chromatography process, as seen on the two peaks obtained, related to elution volumes between 0 and 15.3 ml, and 30.6 and 86.7 ml. A linear gradient concentration of salt was used to elute the samples starting at 0% salt. Since the compounds of interest eluted at early stages, the salt necessary for their displacement from the chromatographic bed was low (peak elution at 0.01M and 0.09M, respectively).

Figure 3.5 shows the protein profile of the samples with the highest papain inhibitory activity. The protein bands range from 6 to 14 kDa. The sample that showed the highest band intensity is the one corresponding to the 30.6 - 86.7 ml elution volume (lane 2), since 1 µl of that sample was loaded compared to 3 µl loaded on the sample collected at an elution volume between 0 and 15.3 ml (lane 3). The difference between the two loaded volumes has to do with previous detection on SDS-PAGE where the first fraction yielded stronger band intensity than

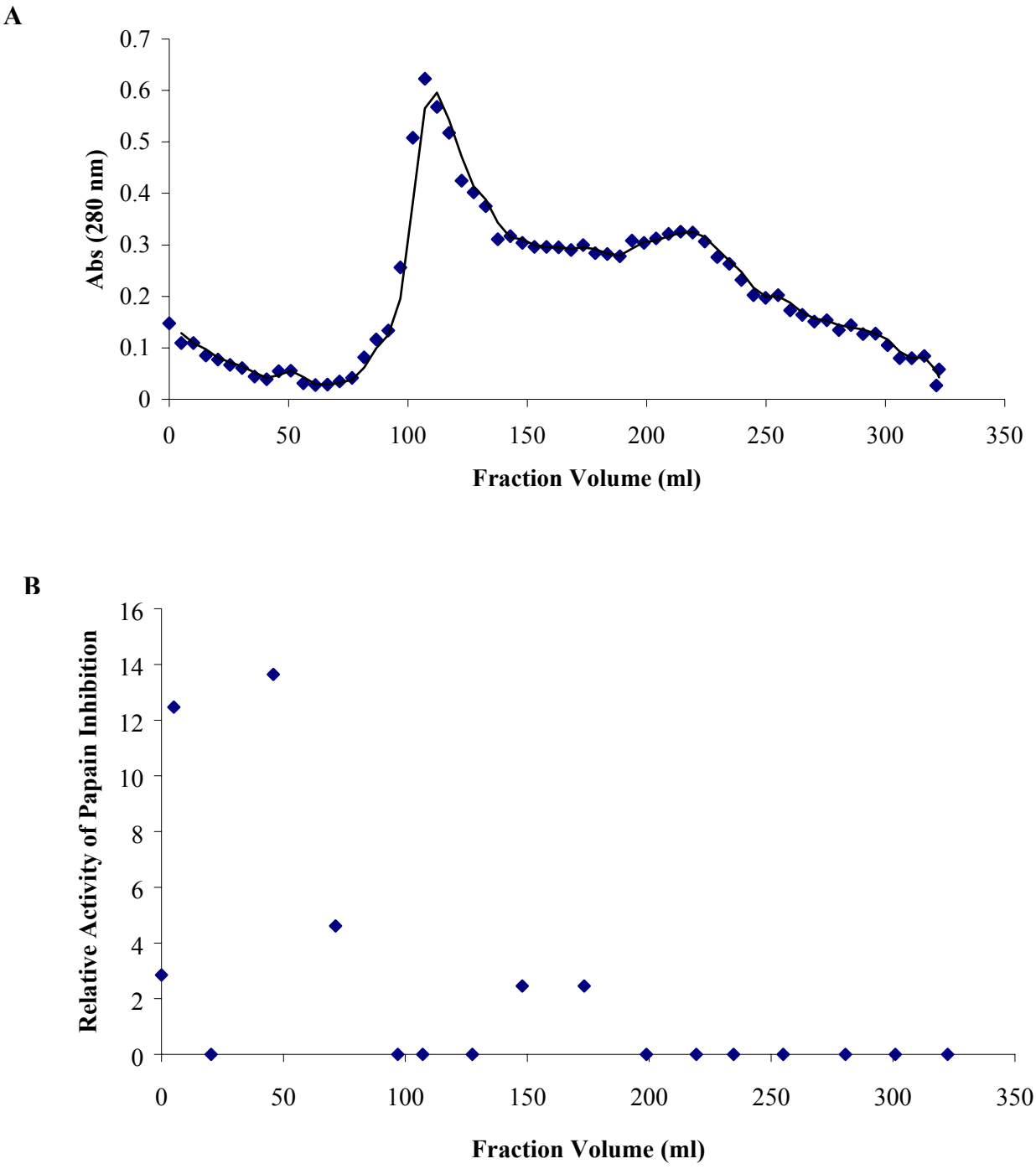

Figure 3.4. Absorbance elution profile (**A**) and papain inhibitory activity profile (**B**) for the samples collected during cation exchange chromatography.

the second fraction mentioned (data not shown). As seen in Figure 3.5, stronger band intensity was seen around 12 kDa for the fraction 30.6-86.7 ml (lane 2), which might be an indication of OCI being present. As stated above, ca. 70 ml were loaded on the CM column. The non-bound portion obtained while loading (ca. 90 ml) was collected and ultrafiltered through a 1 kDa MWCO membrane until a 6-fold concentration was achieved. The ultrafiltered fraction was checked for protein profile. Figure 3.5 (lane 4) shows the protein profile of the ultrafiltered non-bound fraction. It should be noticed that 20 µl were loaded compared to 1 and 3 µl loaded for the eluted samples with papain inhibitory activity. The difference in the volume loaded has to do with the fact that the non-bound fraction was thought to have a lower protein content. Although

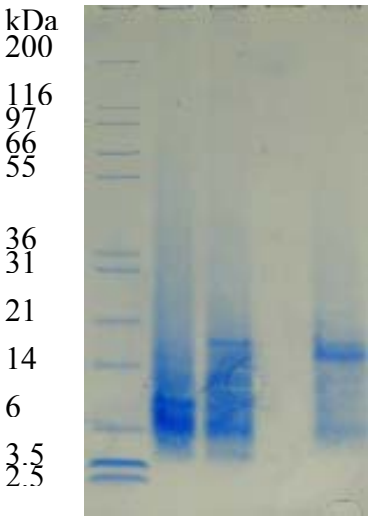

Figure 3.5. SDS-PAGE with Commassie Blue staining for samples pooled after cation exchange chromatography.
Legend:
(1) molecular weight markers (molecular weight (kDa) is shown on the left-hand side of the SDS-PAGE picture)
(2) fraction eluted at 30.6-86.7 ml elution volume (loaded volume: 1 µl)
(3) fraction eluted at 0-15.3 ml elution volume (loaded volume: 3µl)
(4) fraction not bound to the CM column (waste while loading) (loaded volume: 20 µl)

the non-bound fraction showed the same protein profile as the eluted samples with inhibitory activity, the amount of protein was negligible compared to the pooled samples that showed inhibitory activity, as shown in Figure 3.5 (lane 4).

3.3.1.3 Size Exclusion Chromatography

The pooled sample that contained the highest inhibitory activity was loaded on a HiPrep Sephacryl 16/60 S-200 HR column in order to separate the mixture of proteins based on their molecular size.

Figure 3.6 (A) shows the absorbance profile of the size exclusion chromatography. The highest peak was observed at 78-98 ml of elution volume. However, the protein eluting at 78-98 ml had no papain inhibitory activity, which led to the conclusion that the fraction contained no OCI. The fractions that showed inhibitory activity eluted at 62-74 ml and 124-134 ml. Based on the molecular weight of OCI (12 kDa), one can conclude that OCI eluted in the 62-74 ml fractions. The papain inhibitory activity obtained for the fractions eluted at 124-134 ml are most probably due to small peptides that showed non-specific inhibition.

The total protein concentration of the samples collected after size exclusion chromatography is shown in Figure 3.7.

The sample that eluted at 62-74 ml contained ca. 58 μg/ml of total protein whereas the latter fraction contained ca. 29 μg/ml of total protein. Considering that the fraction that is thought to be OCI (62-74 ml elution volume) was 17.5 ml in volume, the protein retained in this fraction corresponded to ca. 1 mg, as shown in Table 3.1. A protein profile is shown in Figure 3.8 for the samples pooled from size exclusion chromatography, which indicated the purity of the protein up to this point.

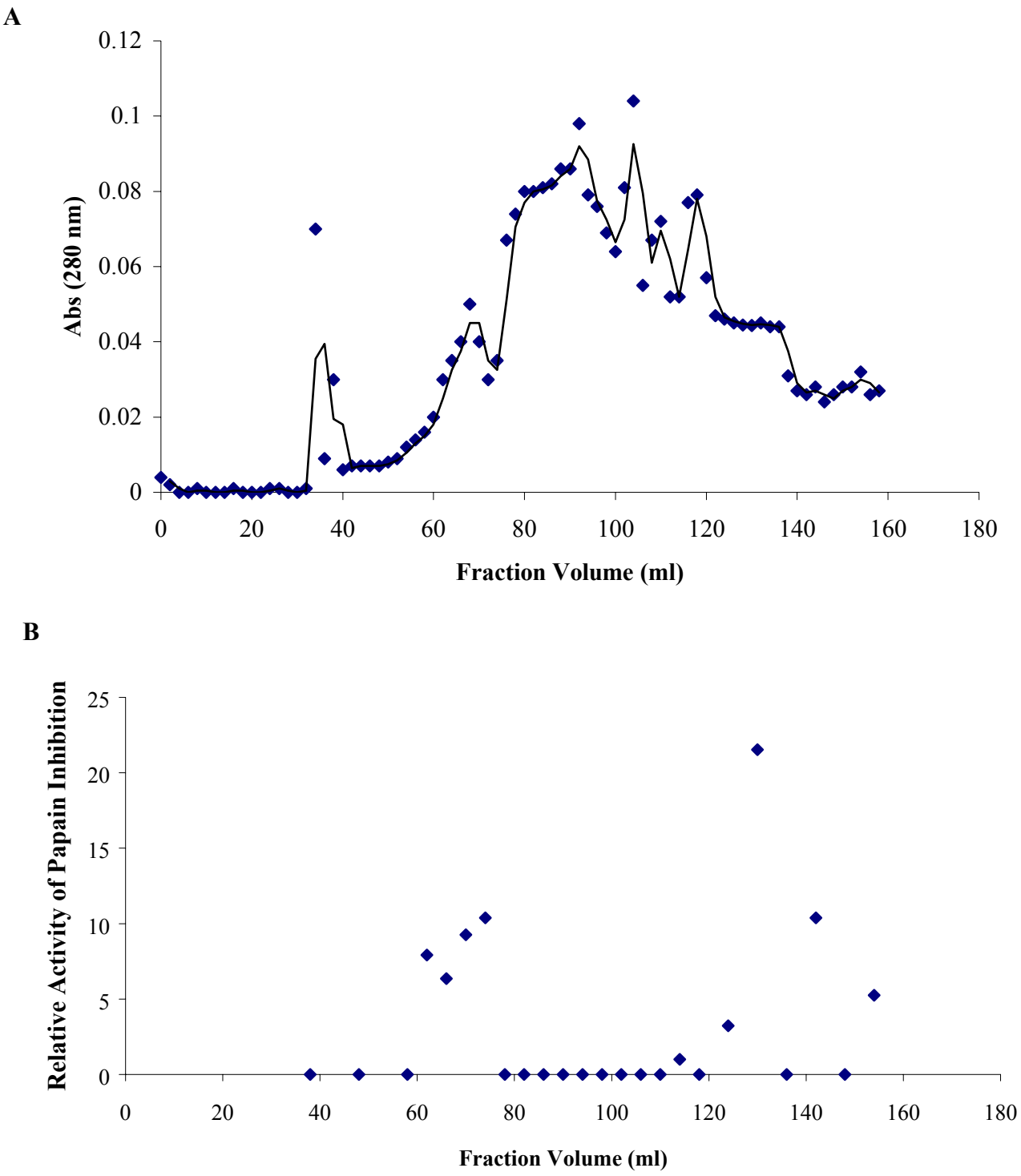

Figure 3.6. Absorbance elution profile (**A**) and papain inhibitory activity profile (**B**) for the samples collected during size exclusion chromatography.

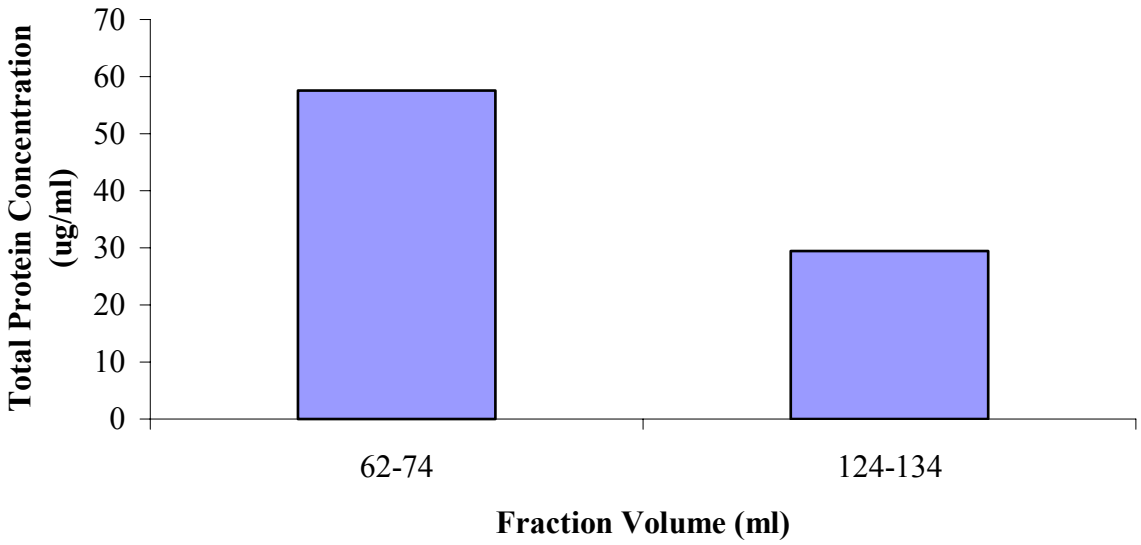

Figure 3.7. Total protein concentration (μg/ml) for the samples pooled from size exclusion chromatography.

As shown in Figure 3.8, three protein bands were seen in fraction volume 62-74 ml, one of them present at 12 kDa, corresponding to OCI. The fraction eluted at 124-134 ml, as expected, showed no band intensity above 6 kDa, although a fainted band was observed below 6 kDa. These findings confirmed that the high papain inhibitory activity observed for these fractions corresponded to small peptides that eluted in the end of size exclusion chromatography. It is worth to mention that this chromatographic step yielded a high dilution on the samples. Comparing the band intensities observed in Figure 3.5 (cation exchange chromatography) with the band intensities observed in Figure 3.8 (size exclusion chromatography), one can notice that the latter are less visible, taking into consideration that the volumes loaded on electrophoresis were similar. Therefore, the samples were freeze-dried in order to continue the purification process. Nevertheless, almost 22-fold increase in specific activity was obtained (Table 3.1) up to this step, which indicates the successfulness of the process.

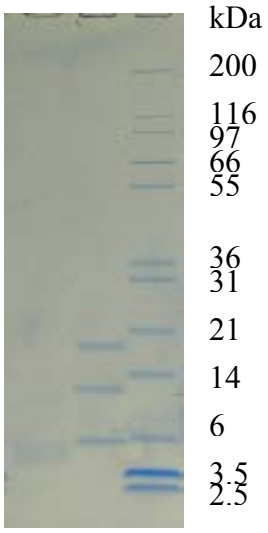

Figure 3.8. SDS-PAGE with Commassie Blue staining for samples pooled after size exclusion chromatography.
Legend:
(1) fraction eluted at 124-134 ml elution volume (loaded volume: 1 µl)
(2) fraction eluted at 62-74 ml elution volume (loaded volume: 3 µl)
(3) molecular eight markers (molecular weight (kDa) is shown on the right-hand side of the SDS-PAGE picture)

3.3.1.4 Anion Exchange Chromatography

To separate the three proteins present in the sample collected at elution volume 62-74 ml from size exclusion chromatography, anion exchange chromatography was carried out with the aim of separating the proteins based on their ionic properties. A HiPrep 16/10 DEAE column was used. The absorbance profile throughout the process is shown in Figure 3.9 (A).

Figure 3.9 (A) shows that the three proteins were effectively separated. Three peaks were observed between 100 and 190 ml, between 192 and 204 ml, and between 300 and 350 ml of elution volume. The papain inhibitory activity assay presented in Figure 3.9 (B) showed that the fraction between 122.5 and 128.8 ml and the fraction between 217 and 248.5 ml of elution volume corresponded to peaks in the papain inhibitory activity. They were eluted at 0.15M and 0.29M of sodium chloride, respectively. OCI was probably present in the fraction that showed

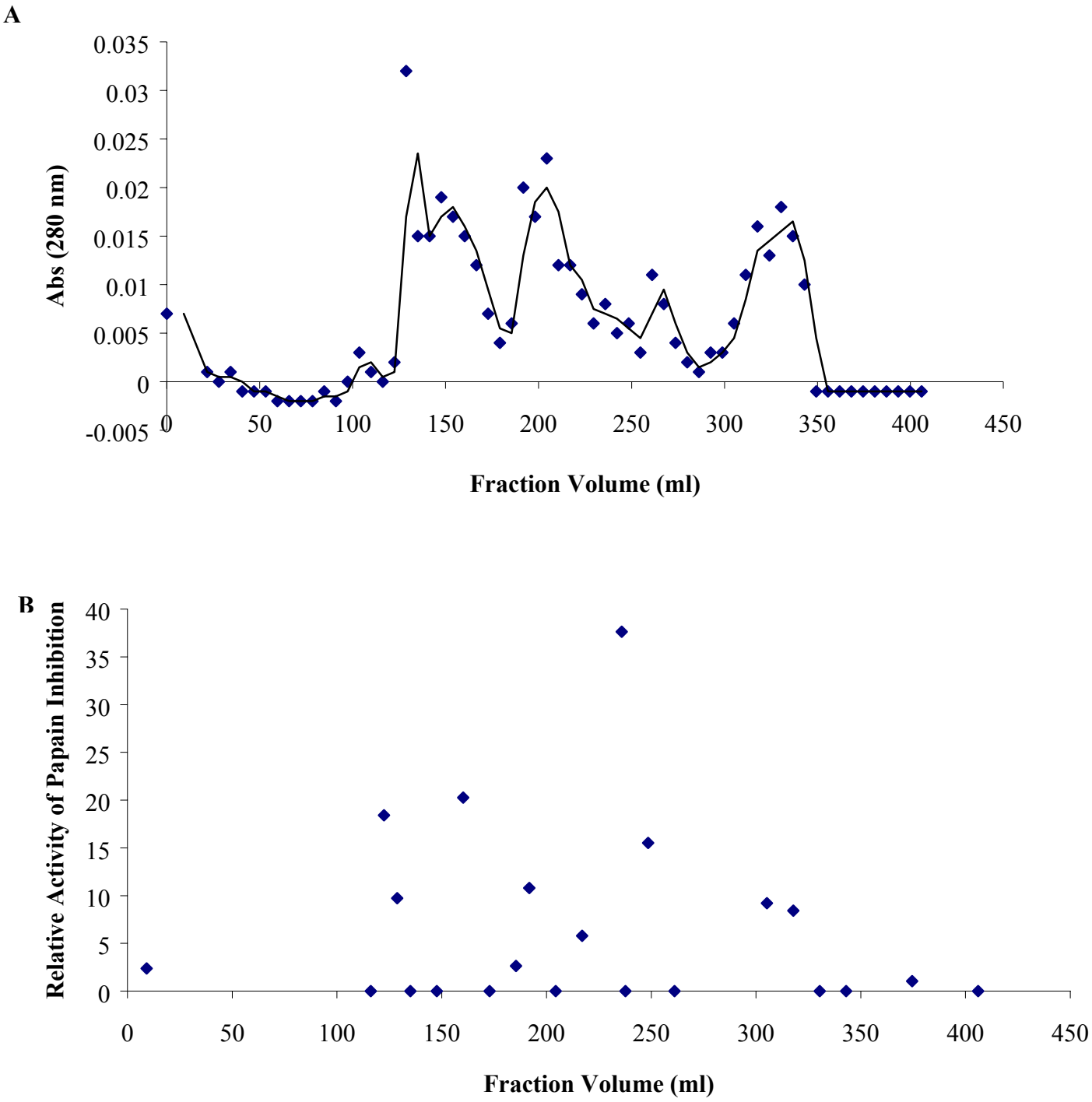

Figure 3.9. Absorbance elution profile (**A**) and papain inhibitory activity profile (**B**) for the samples collected during anion exchange chromatography.

the highest inhibitory activity.

The samples were loaded on an SDS-PAGE gel to check for protein profile of the pooled samples. Figure 3.10 (lane 1) showed a fainted band, indicated with an arrow, at ca. 24 kDa. This result indicated that OCI was pure in this sample but was shown as a dimer. The reason why dimerization occurred was not well understood, since a reducing agent was used while preparing the samples for loading. However, results from papain inhibitory activity showed a high reading for this particular sample, which leads to the conclusion that OCI was present.

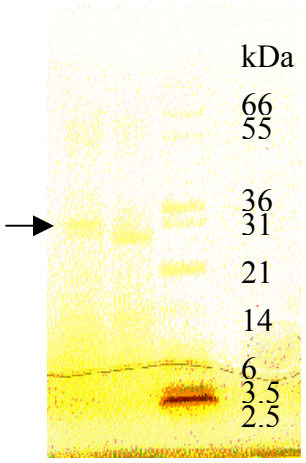

Figure 3.10. SDS-PAGE with silver staining for the pooled samples from anion exchange chromatography.
Legend:
(1) sample eluted at 217-248.5 ml
(2) sample eluted at 122.5-128.8 ml
(3) molecular weight markers (molecular weight (kDa) is shown on the right-hand side of the SDS-PAGE picture)

3.3.1.5 MALDI of OCI

To confirm the presence of OCI in the fraction collected after anion exchange chromatography, the sample (217-248.5 ml elution volume) was further dialyzed and diluted to appropriate concentrations to be analyzed by MALDI-TOF-MS.

Figure 3.11 proved that OCI had been purified, with a molecular weight of 11.5 kDa. Such finding correlates well with data reported by Abe and Arai (1985). The sample was pure, as no other peaks appear on the spectrum. Therefore, one could conclude that while running SDS-PAGE, a dimmer was formed probably due to protein-protein interactions.

Table 3.3 shows the final volume and the total protein content of the fractions collected after anion exchange chromatography, the last purification step in this work.

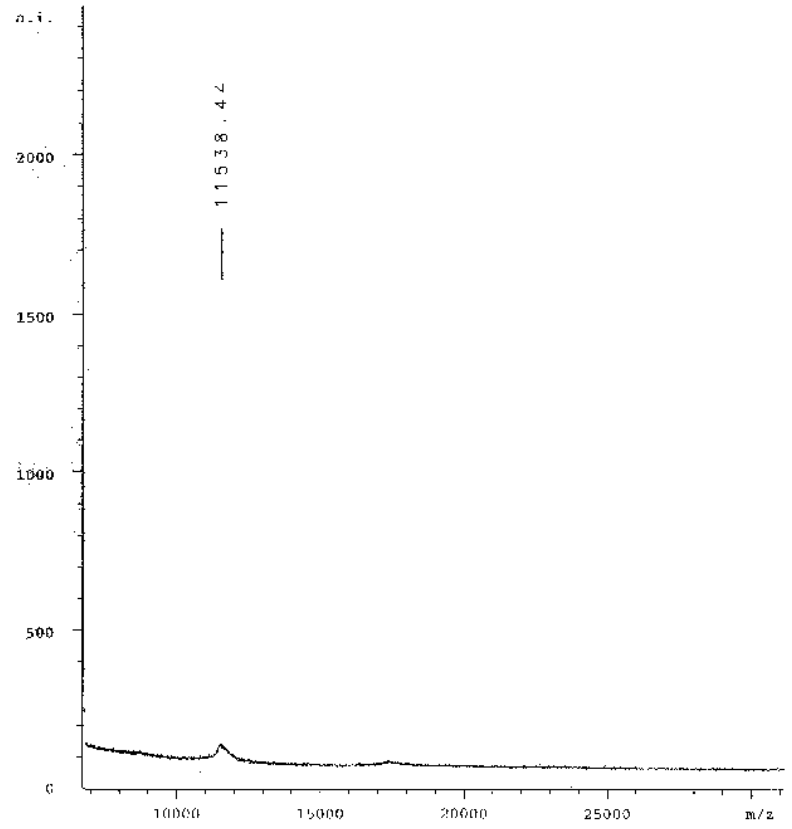

Figure 3.11. MALDI-TOF MS of OCI.

Almost 1 mg of OCI was collected. This finding correlates well with what stated by (Kondo et al., 1989). The authors mentioned that OCI is present in rice seeds at a concentration of 1-4 mg/kg of rice, determined by immunoassay. Considering the fact that the purification

Table 3.3. Final volume and protein content of the samples pooled from anion exchange chromatography.

Fractions collected (elution volume)	Final Volume (ml)	Total Protein Content (mg)
217-248.5 ml	4.750	0.970
122.5-128.8 ml	0.725	0.326

process started with 1 kg of rice bran, one could achieve almost 1 mg of OCI, which indicates that the loss throughout the procedure was not very high. However, a decrease in the specific activity was seen between the two last purification steps, as seen in Table 3.1. The last purification procedure was necessary, since the sample eluted from the previous step, size exclusion chromatography, did not yield a pure compound. Therefore, anion exchange chromatography was carried out to separate the 3 proteins seen, and was successfully accomplished in it's goal of purifying OCI, although with some loss in specific activity. The loss observed might be due to the fact that other proteins present in the fractions obtained before the last purification step (anion exchange chromatography), non-specifically inhibited papain. Proteins like rice Bowman Birk inhibitor might have been finally discarded by the last purification step, therefore decreasing the specific papain inhibitory activity. Overall, the total purification process yielded a 14- fold increase in specific activity of papain inhibition.

3.3.2. Bioavailability Studies

3.3.2.1 Hydrolysis of OCI

Chymotrypsin was used for the hydrolysis of the purified OCI. Although Figure 3.12 is not very clear, a fainted band was seen at molecular weight 12 kDa in lane (2), corresponding to

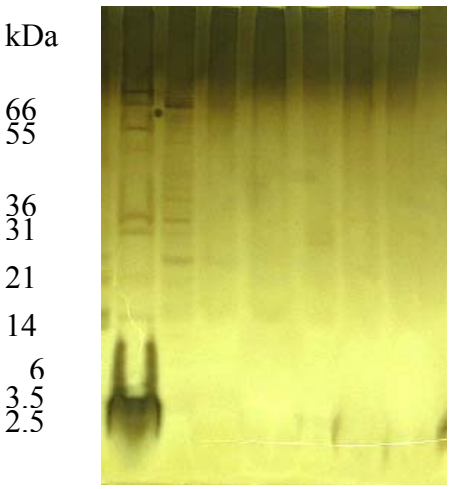

Figure 3.12. SDS-PAGE with silver staining for the OCI hydrolysis process.
Legend:
(1) molecular weight markers (molecular weight (kDa) is shown on the left-hand side of the SDS-PAGE picture)
(2) OCI before hydrolysis
(3) OCI hydrolyzate
(4) control containing chymotrypsin alone (pre-hydrolysis)
(5) control containing chymotrypsin alone (post-hydrolysis)
(6) precipitate obtained after inactivating the mixture of OCI hydrolyzate (post-hydrolysis)
(7) precipitate obtained after inactivating chymotrypsin alone (post-hydrolysis)

OCI. OCI seemed to be present as a dimer, trimer, and tetramer on the sample loaded on lane (2). The bands seen corresponded to OCI, as previously indicated by MALDI-MS analysis (Figure 3.11). The OCI band became almost invisible after the protein had been hydrolyzed in the presence of chymotrypsin, as seen on lane (3). However, smaller peptides corresponding to the hydrolysis of OCI were not clearly seen on the gel. Chymotrypsin was almost invisible due to the very low concentration used (Chymotrypsin:OCI = 1:100). The wells loaded with the precipitated material derived from the heat inactivation of chymotrypsin showed no bands due to the low amount of chymotrypsin, coupled with the fact that it would be difficult to re-dissolve the precipitated chymotrypsin after the heat treatment.

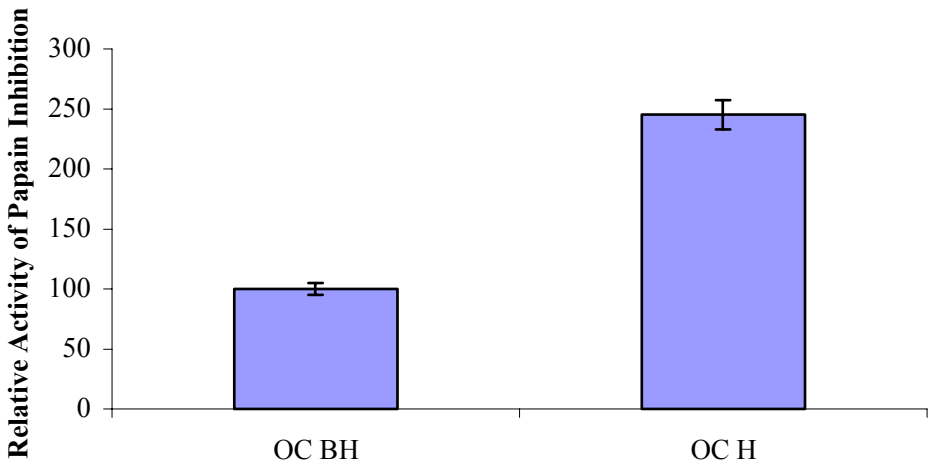

Figure 3.13. Papain inhibitory activity of OCI and OCI hydrolyzate. (Papain inhibitory activity was 100% in OCI before hydrolysis).
Legend:
OC BH: OCI before hydrolysis
OC H: OCI hydrolyzate

To evaluate the effect of hydrolysis on OCI activity, a papain inhibitory activity assay was conducted on OCI and its possible derived peptides. Figure 3.13 shows that the hydrolyzed OCI was more effective against papain than OCI by itself. A 145% increase was achieved in papain inhibitory activity after OCI hydrolysis.

3.3.2.2 Caco-2 Cells Permeability Study

Figure 3.14 shows the papain inhibitory activity obtained in the Caco-2 cells experiment. Comparing the values obtained for the purified OCI, unhydrolyzed OCI did not cross the Caco-2 cell membranes after 3 hours of incubation. Approximately the same inhibitory activity was observed on the sample before incubation and in the apical side of the Caco-2 cells after incubation. Consequently, no inhibition was detected in the basolateral side of those cell membranes. This finding was predictable, since OCI is a relatively large polypeptide to be up taken by the intestinal epithelia. Size is one of the properties involved in intestinal absorption (Egan and Lauri, 2002). Regarding the OCI derived peptides, inhibitory activity was detected in

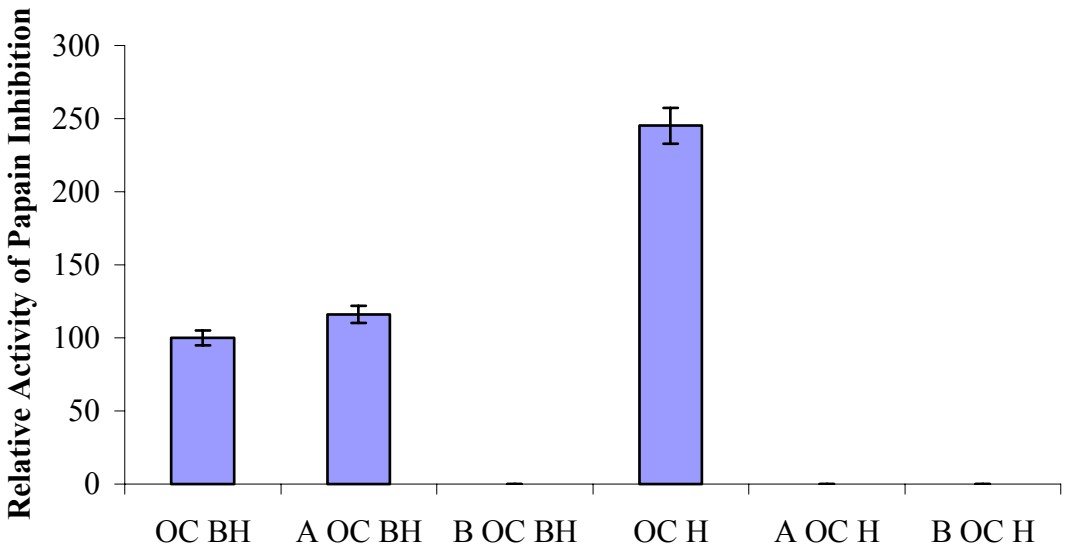

Figure 3.14. Permeability of OCI and OCI derived peptides across Caco-2 cells after 3 hours. (Papain inhibitory activity was 100% in OCI before hydrolysis).
Legend:
OC BH: OCI before hydrolysis
A OC BH: remaining OCI in the apical side of the Transwell membranes
B OC BH: OCI collected in the basolateral side of the Transwell membranes
OC H: OCI derived peptides after hydrolysis
A OC H: remaining OCI derived peptides in the apical side of the Transwell membranes
B OC H: OCI derived peptides collected in the basolateral side of the Transwell membranes

the dosing solutions, as previously observed (Figure 3.13), but no inhibitory activity was detected either on the remaining solution of the apical side or the media collected at the end of the 3 hours of incubation in the basolateral side. As stated by Bretschneider et al. (1999) the transepithelial flux is composed, among other mechanisms, of intracellular accumulation and degradation. There is a possibility that the OCI derived peptides were up taken by the epithelial cells, but once inside the cells, were degraded or simply accumulated. Gabor et al. (2002) studied the delivery of bovine serum albumin in Caco-2 cells by means of conjugating the protein with a plant lectin. They observed that the conjugate followed a pathway characterized by binding to the Caco-2 cell membrane, movement into the cells, and distribution into the lysosomal

compartments. These occurrences were observed within 1 hour. After about 1 to 4 hours proteolytic degradation of the conjugate prevailed. The intralysosomal proteolytic breakdown was followed by diffusion into the cytoplasm and excretion of the low molecular weight products. A tentative explanation for the current results is based on the findings observed by Gabor et al. (2002). The peptides were up taken, accumulated and degraded in the lysosomal compartments of the Caco-2 cells, and the resulting excreted products did not show papain inhibitory activity. Figure 3.15 shows the samples profile when analyzed by MALDI MS. In Figure 3.15 (A), one can notice that no OCI derived peptide was present in the remaining apical media of the Transwells, proving the finding that OCI derived peptides were uptaken by the Caco-2 cells.

Figure 3.15 (B) shows the compounds profile on the basolateral media collected after Caco-2 cells had been exposed to OCI derived peptides for 3 hours. A 5.8 kDa peptide was detected, leading to the conclusion that at least some OCI derived peptides did cross the Caco-2 cells membrane, despite not showing any inhibitory activity towards papain. Due to the impossibility to determine the size of the chymotryptic peptides obtained in the hydrolysis process (Figure 3.12), it was not possible to conclude if the 5.8 kDa peptide seen in the basolateral media of the Caco-2 cells was actually derived from chymotrypsin hydrolysis alone, or it was a result of the action of the proteolytic enzymes present in the Caco-2 cells. Moreover, the Caco-2 cells were only exposed to the OCI derived peptides for 3 hours. Therefore, the possibility that some OCI derived peptides remained accumulated in the lysosomes of the cells and were unable to be excreted still remains. The fact that no papain inhibitory activity was detected in the excreted products of the Caco-2 cells might have to do with the fact that not all the chymotryptic or Caco-2 cells-processed peptides were excreted. A longer exposure of the

OCI chymotryptic peptides to the Caco-2 cells would give an insight of the actual bioavailability of OCI derived peptides. Therefore conclusions regarding the usefulness of OCI as nutraceutical could be drawn.

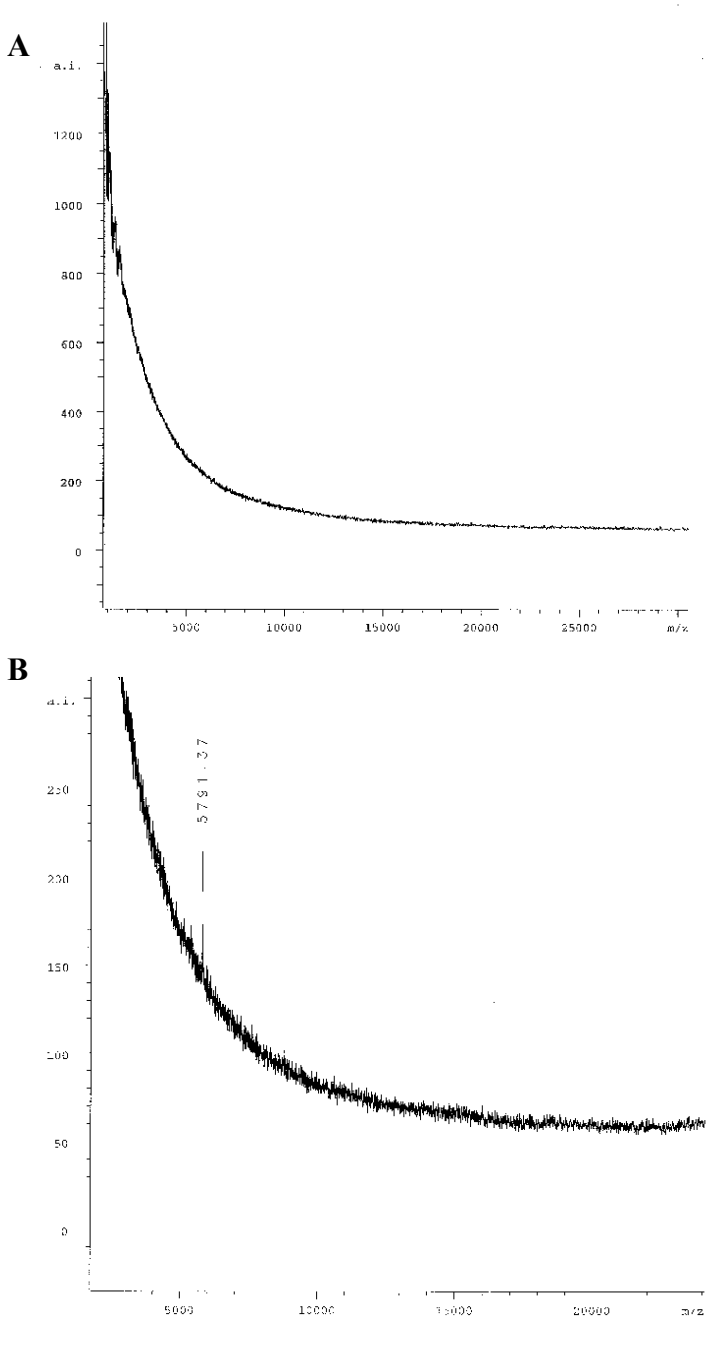

Figure 3.15. MALDI-TOF MS for the apical (**A**) and basolateral (**B**) media collected at the end of the Caco-2 cells bioavailability study.

CHAPTER 4 SUMMARY AND CONCLUSIONS

The present study aimed at recovering and purifying rice cystatin, or oryzacystatin I, from rice bran and testing its oral bioavailability in an attempt to provide scientific evidence that rice cystatin can be used as a functional food ingredient that would have a role of enhancing human health.

Rice bran was homogenized in a 25 mM sodium phosphate buffer containing 0.15 M NaCl at pH 7.0 and sequencely precipitated to remove major proteins present in the rice bran extract. A series of chromatographic steps were carried out in order to separate the remaining proteins based on their ionic properties as well as molecular size properties. Oryzacystatin I was successfully recovered from the rice bran homogenate and yielded an amount of approximately 1 mg of pure protein out of 1 kg of rice bran. A 14-fold increase in specific activity was observed throughout the purification process.

Oryzacystatin I bioavailability was studied using Caco-2 cells. The purified oryzacystatin I was hydrolyzed by chymotrypsin. The chymotryptic peptides yielded a higher papain inhibitory activity compared to oryzacystatin I (145% increase). Our results showed that unhydrolyzed oryzacystatin I did not cross the cell membrane. The chymotryptic peptides were uptaken by the cells. Only one peptide crossed the cell membrane and was detected on the basolateral side of the Caco-2 cells. The oryzacystatin I peptide that crossed the Caco-2 cell membrane showed no papain inhibitory activity. At this point we may not speculate on the outcome of the peptide until a longer incubation is performed. A tentative explanation is a potential interaction between the peptides and the lysosomes in the Caco-2 cells.

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VITA

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