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Calanus finmarchicus as
a Potential Basic Feed
Ingredient

Protease Activity and Encapsulation
Strategies

Thesis for the degree of Philosophiae Doctor

Trondheim, September 2008

Norwegian University of Science and Technology
Faculty of Natural Science and Technology
Department of Biotechnology



NTNU

Norwegian University of Science and Technology

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ISBN 978-82-471-1174-1 (printed ver.)
ISBN 978-82-471-1175-8 (electronic ver.)
ISSN 1503-8181

Doctoral theses at NTNU, 2008:241

Printed by NTNU-trykk

Acknowledgments

The work presented in this thesis was carried out at the Department of Biotechnology at The Norwegian University of Science and Technology (NTNU) in the period from 2004 to 2008, and the work has been a part of the strategic university program, Codtech.

Professor Kurt I. Draget has been my supervisor during this study, to whom I am grateful for useful discussions and co-operations throughout these years.

I would also want to thank Prof. Olav Vadstein, Prof. Kjell M. Vårum and Prof. Turid Rustad for advices and discussions. I also need to thank my parents who have supported me all my years.

I want to mention my friends in Trondheim: Kjetil Midthun, Hugo Hammer, Håvard Berland, Steinar Kragset, Torfinn Eriksen and Jostein Eriksen. You have all been good supportive friends, and have made my time in Trondheim more enjoyable.

Finally, I also need to thank my colleagues Ingrid A. Draget, Ann-Sissel Ulseth, Ingvild J. Haug, Jonhard Eysturskard, Aslak Einbu, Torunn Forberg, Lena S. Sal, and everybody else, who have helped me in addition to being great company at coffee breaks and lunches.

Summary

The purpose of this thesis was to study *Calanus finmarchicus* as a potential basic feed ingredient for cod larvae. The focus was on proteolytic activity in *C. finmarchicus*, release of proteinous components from Ca-alginate encapsulated *C. finmarchicus*, and fish gelatin (FG) as an alternative gelling agent to alginate. In addition, chitosan coating of alginate beads as a method for controlling the release of a model molecule was investigated.

The proteolytic activity in crude extracts of *C. finmarchicus* was studied in order to get a picture of the protease activity and protease classes responsible for the degradation of the protein. The general proteolytic activity was highest at pH 7, and the overall temperature optimum was at 50 °C. Serine and metallo proteases were found to be responsible for the proteolytic activity at neutral and alkaline conditions, whereas aspartic protease were dominant at acidic conditions. Cystein proteases did not seem to be present in any significant amounts.

Homogenized *C. finmarchicus* was encapsulated in Ca-alginate and the release of amino groups and intact proteins was investigated. The highest release at neutral conditions was at 50 °C, however, it was surpassed with time by 40 °C probably due to protease instability. The release of proteins at pH 5 increased with increasing ionic strength, which was probably because of shielding effects as well as reduction in the entropic driving force. However, some of the release may be due to increased solubility of salt soluble proteins. In addition, the release was also found to be highly affected by pH. The highest release was at alkaline conditions, which was probably due to decreased attraction between protein and the negatively charged alginate network, in addition to increased protein solubility.

The suitability of FG (fish gelatin) as an alternative gelling agent for *C. finmarchicus* was studied. FG in solution was readily degraded by crude proteases at room temperature and above. Cold set gels of 10 % (w/v) FG were similarly degraded at temperature between 4 and 10 °C, which suggested that FG would not be suitable in feed applications in the presence of active *Calanus* proteases.

Blue dextran was used as a model molecule to study the release from Ca-alginate capsules. The release decreased with decreasing degree of acetylation of the chitosans, as

expected. Hen egg white lysozyme seemed to destabilize the coating with $F_A=0.4$ leading to a small increased release of blue dextran, whereas the coating with $F_A=0.12$ inhibited the release probably due to non-productive binding of lysozyme to the chitosan layer.

Table of contents

Acknowledgments.....	i
Summary	ii
Table of contents.....	iv
About the papers	vi
List of papers.....	vi
1. Introduction.....	1
1.1. The scope	1
1.2. Cod farming	1
1.2.1. Cod larvae	2
1.2.2. Live feed	3
1.3. <i>Calanus finmarchicus</i>	4
1.4. Proteolytic enzymes.....	6
1.4.1. Enzymes.....	6
1.4.2. Proteases and protease classes	7
1.4.3. Serine proteases	8
1.4.4. Cysteine proteases.....	9
1.4.5. Aspartic proteases	9
1.4.6. Metallo proteases	9
1.5. Control of proteolytic activity.....	10
1.5.1. Temperature and pH	10
1.5.2. Protease inhibitors.....	10
1.6. Alginate.....	11
1.7. Fish gelatin (FG).....	13
1.8. Chitosan	14
2. Experimental aspects	17
2.1. The raw material	17
2.2. Analytical methods for unpublished results.....	18
2.2.1. Measurements of trypsin-like activity	18
2.2.2. Chromatography methods.....	18
3. Results and general discussion.....	20
3.1. Protease activity in <i>C. finmarchicus</i>	20
3.1.1. The biochemical composition of <i>C. finmarchicus</i>	20
3.1.2. Proteolytic activity in <i>C. finmarchicus</i>	21
3.1.3. Protease classes in <i>C. finmarchicus</i>	23
3.1.4. Trypsin-like activity in <i>C. finmarchicus</i>	24
3.1.5. Partial purification of proteases in the <i>C. finmarchicus</i>	26
3.2. Release of proteinous components from alginate- <i>Calanus</i> particles.....	28
3.2.1. Release of amino groups.....	28
3.2.2. Release of protein	31
3.2.3. Effect of pretreatment on release of protein and amino groups.....	33
3.3. Proteolytic degradation of FG with proteases from <i>C. finmarchicus</i>	35
3.3.1. Degradation of FG solution	35
3.3.2. Proteolytic degradation of FG gels	36
3.4. Some release properties of chitosan coated alginate capsules	39

3.4.1.	Release of hemoglobin.....	39
3.4.2.	Release of blue dextran	41
3.4.3.	Effect of lysozyme on BD release	43
4.	Concluding remarks and future perspectives	47
	List of symbols and abbreviations	49
	References.....	50

About the papers

This thesis consists of 4 scientific papers and an extended summary. Some relevant data not covered by the papers are included in the summary.

Paper 1 and 2 are published and have appeared in the journals. Paper 3 is in press, whereas paper 4 is in prep. The papers are presented chronologically.

List of papers

1. Solgaard, G., Standal, I. B., and Draget, K. I. (2007). Proteolytic activity and protease classes in the zooplankton species *Calanus finmarchicus*. *Comparative Biochemistry and Physiology, Part B* 147: 475-481.
2. Solgaard, G., Thorsen, K. H., and Draget K. I. (2008). Encapsulation of a proteolytically active novel bioproduct; controlling the release of proteinous components. Accepted for publication in *Food and Bioproducts Processing*.
3. Solgaard, G., Haug, I. J., and Draget, K. I. (2008). Proteolytic degradation of cold-water fish gelatin solutions and gels. *International Journal of Biological Macromolecules*, 43: 192-197.
4. Solgaard, G. and Draget, K. I. (2008). Release of a model macromolecule from chitosan-coated alginate capsules; effect of F_A and lysozyme. In prep.

1. Introduction

This work forms part of the strategic research program “Codtech: A process oriented approach to intensive production of marine juveniles with main emphasis on cod”, where the objective has been to optimize the key components of the post-rotifer feeding regime of cod larvae based on their requirements, with main emphasis on methods for early weaning.

1.1. The scope

The initial scope of this thesis was to study methods for production of feed particles for cod larvae with *C. finmarchicus* as a major basic feed constituent. The challenge was to develop feed particles of appropriate size and with optimized content of nutrients and stability. In addition, the feed should be appetizing and result in low mortality rate.

Initial experiments with zooplankton as raw material showed that the major problem was poor protein stability in the feed. Therefore, the revised goals became to characterize the proteolytic activity in the raw material in order to control the catabolic processes (Paper 1), in addition to study the release of proteinous components from feed particles (Paper 2). Fish gelatin, as an alternative gelling agent was studied since it may also act as a protein and energy source (Paper 3). In addition, coating of alginate capsules was studied as a method to further control the release of molecules from alginate particles (Paper 4).

1.2. Cod farming

Atlantic Cod (*Gadus Morhua*) is a common food finfish belonging to the family *Gadidae* (Fig. 1.1).

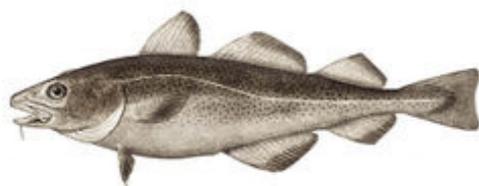


Fig. 1.1 Atlantic cod (*Gadus Morhua*).

Cod is a popular food fish with a mild flavor, low fat content and a dense white flesh that flakes easily. Cod liver oil is also a valuable product of cod, which is highly exploited as an important source for omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in addition to vitamin A and D.

Harvesting of cod has been an important food supply in Norway and many other countries. Recently however, the harvesting has reached an upper limit and further increase in fishing of this species may lead to a drastic reduction of the fish stock [1, 2]. It is therefore important to supply the market with farmed cod so that the demand for this species can be fulfilled without exhausting the natural source [3]. At present, the countries which are involved in cod farming is Norway, Iceland, UK, US and Canada [4]. The first attempt in cod farming in Norway was performed in Flødevigen in the 1880s [4, 5], but it was not until the end of the 1970s that cod farming caught serious interest. Since then there has been many attempts to establish successful cod farming businesses in Norway and abroad. However, the production has been confronted by low cod prices and high production costs. Lately however, the production of farmed cod has increased and there is a renewed optimism in the business.

1.2.1. Cod larvae

Cod larvae have traditionally been considered to demand live feed. The reason for this was the belief that the larvae's digestive system contained an inadequate amount of digestive enzymes, and that active enzymes from the live feed was needed to process the feed successfully. More recently however, it has been shown that all digestive enzymes, except stomach enzymes, seem to be present in marine fish larvae during the first days of feeding, and it is now established that larvae are capable of digesting formulated feed from the beginning of start feeding [6, 7].

The first 30 days post hatching (DPH) is the most vulnerable period in cod farming. The feed intake the first two DPH is solely endogenous, whereas the following days consist of a mixed-feeding mode where nutrients are extracted both from the surroundings and intrinsically from the egg yolk. Rotifers are the main feed source in this mixed-feeding period (see Fig. 1.2.). The larvae start to feed fully exogenously after about 15 DPH, at which point the larvae begin feeding on *Artemia*. After 30 DPH

(around metamorphosis), the larvae are more developed and dry formulated feed is introduced. A more extensive presentation of all aspects related to production of cod and other cold-water fish is found in Moksness [8].

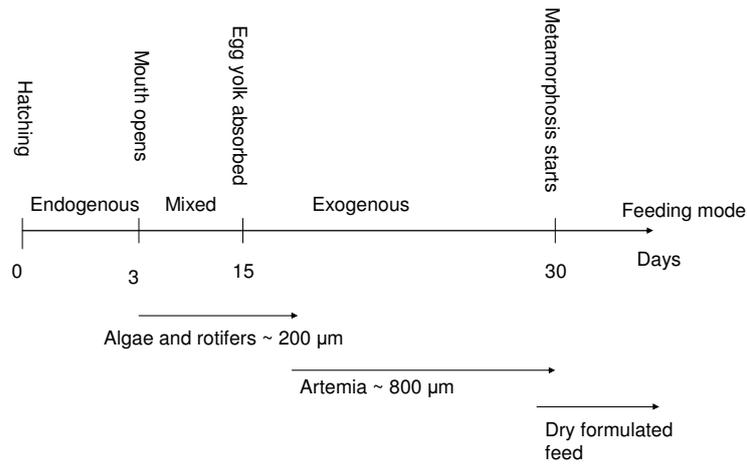


Fig. 1.2. Development of cod larvae and feeding strategy.

1.2.2. Live feed

As depicted in Fig. 1.2 the major food components in first feeding of cod larva are microalgae, rotifers and Artemia [8]. Microalgae (*Isochrysis galbana*, *Tetraselmis sp.*) are not strictly necessary for all species during larval feeding, but it is well known to enhance production yields and quality of many species. The algae contribute primarily with lipid such as DHA (docosahexaenoic acid) and n-HUFA (highly unsaturated fatty acid) in general.

Rotifers (*B. plicatilis*, *Brachionus rotundiformis*) are a major component in first feeding and there are a wide variety in sizes and nutritional content. The nutritional value is very dependent on cultivation method and use, but it contributes with energy, protein, essential fatty acids, vitamins and minerals.

Artemia Naupli (*Artemia franciscana*) is used in first farming of marine fish species, and is considered to have much more stable nutritional content (protein and lipid) than

rotifers. However, the level of DHA and EPA is low in *Artemia*, and thus has to be enriched with this fatty acid before it is adequate as live feed for marine fish larvae. Recent research suggests that DHA is more important nutritionally than EPA, and the ratio DHA:EPA is considered to be of most importance [6, 7].

Marine cold-water fish larvae need high proportions of n-HUFA to meet their requirements for growth and development [8]. In addition, a sufficient amount of protein is necessary to provide the fish with energy and substrate for growth [8]. Enriched *Artemia* works as main feed in cod juvenile production, but the culturing of this species is both expensive and labor intensive. An alternative larva feed to replace the *Artemia* would be beneficial. Thus, the study of other possible species has been initiated, and in this thesis, the focus has been on the zooplankton species *C. finmarchicus* as a possible basic feed ingredient.

1.3. *Calanus finmarchicus*

C. finmarchicus (in Norwegian called “raudåte”) is a zooplankton species that is present in enormous amounts in the North Atlantic and the Arctic Ocean [9, 10]. It can be easily harvested in large quantities by trawling. However, the harvest technology is still not optimized, and future technology will probably improve the yield significantly. The species should be regarded as a potentially valuable marine resource. It is estimated that one would be able to supply most of the marine farming industry with enough feed by harvesting only a small fraction of the population of *C. finmarchicus*.

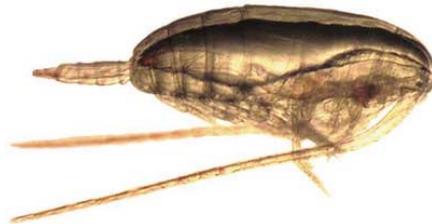


Fig 1.3 One individual of the *C. finmarchicus* zooplankton species [11].

C. finmarchicus contains mainly lipids, protein, chitin and lipids. The lipids include triglycerides, wax esters and phospholipids [12], which contain important fatty acids such DHA and EPA [13]. Commercial products such as *Calanus* powder and *Calanus* oil are

available (www.calanus.no), and they have been designed as supplements in marine feed formulas. Drying of *C. finmarchicus*, to produce *Calanus* powder, may be achieved by applying heat or by a gentler freeze-drying process, whereas pressing of *C. finmarchicus* is performed to extract the *Calanus* oil, which is regarded as valuable due to its high content of omega-3 acids. The chitin fraction in *C. finmarchicus* is low (2-3 %), however, the species may be considered as a chitin source in the future due to the massive amounts. The byproducts after lipid and protein extraction may be used to extract chitin for research and commercial use.

The biochemical composition of some copepod stadia of *C. finmarchicus* may be considered beneficial with respect to feed. The amino acid composition of the marine protein and the lipid profile of the marine lipid should be well suited in fish feed formulations [14]. Copepods in general constitute a major part of the diet of the fish larva in the natural pelagic food chain and it is believed that copepods should meet the requirements of marine fish larva [13]. The traditional feed used in marine fish farming has been live feed, such as *Artemia* and rotifers [15], which have been cultivated in tanks *in situ* prior to feeding. The omission of this primary step may be accomplished through the production of a storable pre-hydrated fresh feed, which could be procured from another location.

A possible feed for marine larvae could be encapsulated *C. finmarchicus* particles. A widely used encapsulated agent of marine origin is alginate, and this marine polysaccharide gives temperature stable gel particles (see section 1.6), which may serve as a matrix for homogenized *C. finmarchicus*. The encapsulation procedure is simple and makes it very easy to incorporate other additives as vitamins, minerals, oil and protein (e.g. soy protein powder). However, it is important to control the stability and release of the nutrients from such particles. In addition, sinking rate and acceptance must be known and the particles must be tested in start feeding experiments before any commercial production can be launched.

A feed acceptance experiment performed in 2003 showed that alginate encapsulated *C. finmarchicus* was ingested and digested by cod larvae (unpublished work). The larvae increased in weight when this feed was fed to the larvae in the period 20-27 DPH. This

suggested that alginate encapsulated *C. finmarchicus* could be a potential novel larval feed and that further research was justified.

The previous positive result was followed by a start feeding experiment in the end of 2004, where alginate encapsulated calanus were fed to larvae 15-30 DPH. This experiment exhibited very high mortality and later experiments showed a decreased protein content in the feed of which 30 % was lost after thawing (unpublished results). This percentage roughly equals the amount of water-soluble protein in the in *C. finmarchicus*. Additionally, protease activity in water extracts was later found to be significant. Hence, it became obvious that the proteins were easily lost through diffusion out of the alginate network. The proteolytic activity would greatly facilitate this, since especially small peptides and free amino acids would quickly diffuse out the alginate network. Thus, the main challenge was to control this proteolytic activity and the release of proteinous components from the feed particles.

1.4. Proteolytic enzymes

1.4.1. Enzymes

Enzymes are biological macromolecules that catalyze biochemical reactions [16]. Most of these are proteins, but some RNA molecules have more recently been discovered to have catalytic properties [17] and are often called ribozymes. In addition, synthetic molecules called artificial enzymes also display enzyme-like catalysis [18, 19].

In enzymatic reactions, the substrates (reactants) are processed by the enzyme and converted in an accelerated way into products. Like all catalysts, enzymes work by lowering the activation energy (E_a or ΔG^\ddagger) for a reaction, and thereby accelerating the rate of the reaction dramatically. Most enzyme reaction rates are millions of times faster than non-catalyzed reactions. Enzymes are not consumed by the reactions that they catalyze, nor do they alter the equilibrium of these reactions. However, enzymes do differ from most other catalysts by being much more specific.

The term activation energy was introduced in 1889 by Svante Arrhenius [20], and was defined as the energy needed to be overcome in order for a chemical reaction to proceed. The activation energy is the height of the energy barrier separating two minima of potential energy of the reactants and of the products of a reaction. For chemical reactions

to have noticeable rate, there should be a significant number of molecules with the energy equal or greater than the activation energy.

The Arrhenius equation (see equation 1 below) is an expression that shows the dependence of the rate constant k of chemical reactions on the absolute temperature T and activation energy E_A , where A is the pre-exponential factor or simply the pre-factor and R is the gas constant. It can be seen that either increasing the temperature or decreasing the activation energy (by catalysts or enzymes) will result in an increase in rate of reaction. E_A of enzymes is useful in order to predict the breakdown of macromolecules in e.g. *C. finmarchicus* during storage and processing at given temperatures.

$$k = Ae^{\frac{-E_A}{RT}} \quad (1)$$

Taking the natural logarithm of the Arrhenius equation yields:

$$\ln k = \ln A - \frac{E_a}{R} \frac{1}{T} \quad (2)$$

When a reaction has a rate constant, which obeys the Arrhenius equation, a plot of $\ln k$ versus T^{-1} gives a straight line. The slope and intercept can be used to determine E_A and A .

1.4.2. Proteases and protease classes

Proteolytic enzymes are enzymes that hydrolyze peptide bonds (amide bond) in proteins and oligopeptides as depicted in Fig. 1.4 [16]. Alternative names of proteolytic enzymes are proteases, peptide hydrolases, peptidases or proteinases. In this thesis, all enzymes that hydrolyze peptide bonds will be referred to collectively as proteases.

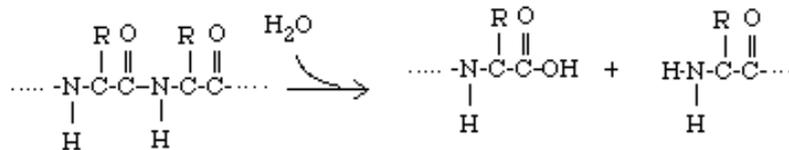


Fig. 1.4 Hydrolysis of a peptide bond. R represents an amino acid side group.

Proteases have been intensively studied because of their importance in biological systems, and the number of different proteases have been subsequently identified and

characterized. The various proteases use different strategies to hydrolyze peptide bonds, and it is useful to classify these enzymes based on the reaction mechanism. Most proteolytic enzymes are either classified as serine proteases, cysteine proteases, aspartic proteases or metallo proteases depending on the nature of their active sites [21], and the reaction mechanism of these are outlined briefly in section 1.4.3-1.4.6.

Proteases are also termed as either endo- or exo-proteases. The endo-proteases hydrolyze peptide bonds internally in peptide chains and the resulting products are oligopeptides. Exo-proteases, on the other hand, hydrolyze peptide bonds near the terminal ends, and the products of this activity are free amino acids, tri- and di-peptides.

1.4.3. Serine proteases

Serine proteases are a class of proteases, which have a serine residue at their active site, and are known to bind covalently to the substrate [16, 21]. Serine proteases are mostly active at neutral and alkaline conditions, and a wide range of functions in the body, including blood clotting, immunity, and inflammation, as well as contributing to digestive enzymes in both prokaryotes and eukaryotes.

Serine proteases are grouped into clans that share structural homology and then further sub grouped into families that share close sequence homology. The major clans found in humans include the chymotrypsin-like, the subtilisin-like, the alpha/beta hydrolase and signal peptidase clans.

The main unit in the catalytic mechanism in the chymotrypsin and subtilisin clan enzymes mentioned above is the catalytic triad [21]. The triad is located in the active site of the enzyme, where catalysis occurs, and is preserved in all serine proteases. The triad is a coordinated structure consisting of three essential amino acids: histidine, serine (hence the name "serine protease") and aspartic acid. Located very near one another near the center of the enzyme, these three key amino acids each play an essential role in the cleaving ability of the proteases.

During catalysis, an ordered mechanism occurs in which several intermediates are generated. The catalysis of the peptide cleavage can be regarded as a ping-pong catalysis, in which a protein binds a product is released (the N-terminus part of the protein), a water molecule binds and another product is released (the C-terminus part of the peptide).

1.4.4. Cysteine proteases

Cysteine proteases have a common catalytic mechanism that involves a nucleophilic cysteine thiol in a catalytic triad [22]. The first step is de-protonation of a thiol in the enzyme's active site by an adjacent amino acid with a basic side chain, usually a histidine residue [21]. The next step is nucleophilic attack by the de-protonated cysteine's anionic sulfur on the substrate carbonyl carbon. In this step, a fragment of the substrate is released with an amine terminus, the histidine residue in the protease is restored to its de-protonated form and a thio-ester intermediate linking the new carboxy-terminus of the substrate to the cysteine thiol is formed. The thio-ester bond is subsequently hydrolyzed to generate a carboxylic acid moiety on the remaining substrate fragment, while regenerating the free enzyme.

1.4.5. Aspartic proteases

Aspartic proteases utilize an aspartic acid residue for catalysis of their peptide substrates [16]. They typically have two highly conserved aspartates in the active site and are optimally active at acidic pH.

While a number of different mechanisms for aspartic proteases have been proposed, the most widely accepted is a general acid-base mechanism, which involves coordination of a water molecule between the two highly conserved aspartate residues [21]. One aspartate activates the water by abstracting a proton, enabling the water to attack the carbonyl carbon of the substrate scissile bond generating a tetrahedral oxy-anion intermediate. Rearrangement of this intermediate leads to protonation of the scissile amide.

1.4.6. Metallo proteases

The metallo proteases have metal ions as cofactors at their active site, and zinc ions (Zn^{2+}) are the most common ion involved in such mechanism. Carboxypeptidase A and B and thermolysin are the most studied representatives of this class [23]. The metal ion is important in the mechanism and provides a strong electrophilic "pull" to assist in the attack by the water molecule [21]. The enzyme has a water molecule coordinated to the fourth tetrahedral site (the other ligands to the metal are two histidines and a glutamic acid in carboxypeptidase A and thermolysin). This water molecule may be displaced upon

coordination of the substrate carbonyl to the metal atom; however, it is believed to remain at the active site. It has been suggested that it may remain coordinated to metal atom at least in a transition state. The water molecule is hydrogen bonded to a glutamic acid residue. That carboxyl group serves as a general base to remove a proton and assist the attack of the same water molecule on the peptide carbonyl. Again, a proton must be transferred to the leaving nitrogen atom and this could be derived from the glutamic acid. Hence, the glutamic acid would be acting as a “shuttle” in analogy to one of the catalytic groups in the aspartic proteases and to the histidine in the serine and cysteine proteases.

1.5. Control of proteolytic activity

Proteolytic activity can be controlled or eliminated by changing external factors such as temperature and pH as well as addition of inhibitors or denaturants. Such treatments may lead to reversible or irreversible deactivation of the proteases. For most purposes in food science, a total irreversible deactivation of proteolytic activity may be acceptable or even desirable in order to keep a constant protein level and otherwise a stable product. For marine larva feed production, however, a low level of proteolytic activity may be beneficial since it should make the feed more digestible for young individuals, which still have sub-optimal digestive system compared to mature individuals even though some digestive enzymes are present [7].

1.5.1. Temperature and pH

Temperature deactivates protein by denaturation, thus destroying the three dimensional structure which is essential for the catalytic properties. This treatment may or may not lead to irreversible deactivation of the enzymes, depending on the length and harshness of the heating process.

1.5.2. Protease inhibitors

Protease inhibitors are molecules, which binds to proteases and forms an enzyme-complex that stops or lowers proteolytic activity, either irreversibly or reversibly [21]. Irreversible inhibition occurs if the inhibitor molecule binds strongly (covalently or non-covalently) to the proteases, while reversible inhibitors are more loosely bound and dissociates more easily from the enzyme-inhibitor complex. An inhibitor is further

characterized as competitive or non-competitive. A competitive inhibitor binds to the active site of the enzyme preventing substrates to attach. This type of inhibition can be reduced by increasing the substrate concentration. In non-competitive inhibition, both substrate and inhibitor bind to the enzyme, and this kind of inhibition is not affected by substrate concentration.

Specific inhibitors can be used to inhibit the different protease classes [21]. Serine proteases are inhibited by e.g. AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride), which is a water soluble, irreversible serine protease inhibitor. Metallo proteases are inactivated by removing the metal ion, which is associated with the active site. EDTA (ethylene diamine tetraacetic acid) binds di- and trivalent ions, and is thus capable of inhibiting metallo proteases. Cysteine proteases are inhibited by NEM (N-Ethylmaleimide), and aspartic proteases are inhibited by Pepstatin A.

These inhibitors have been used to identify the different protease classes in *C. finmarchicus* by comparing the general proteolytic activity in crude extract with and without inhibitor (Paper 1).

1.6. Alginate

Alginate is a marine polysaccharide built up by mannuronic acid (M) and guluronic acid (G), which are linked by β -1,4 glycosidic linkages (Fig. 1.5) [24].

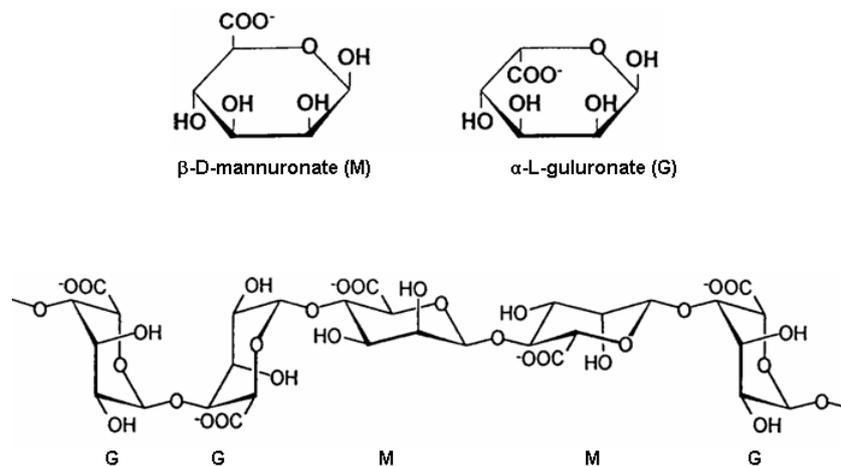


Fig.1.5 The building blocks of alginate and the alginate structure [24].

The fractions of M and G, and their distribution, are determined by ^1H NMR [25, 26]. The main source of alginate is the brown alga (e.g. *Laminaria digitata*, *Laminaria hyperborea*), however, some alginates are of bacterial origin [24]. The properties of the alginates depend highly on its composition and the sequential structure, which again depend on the source.

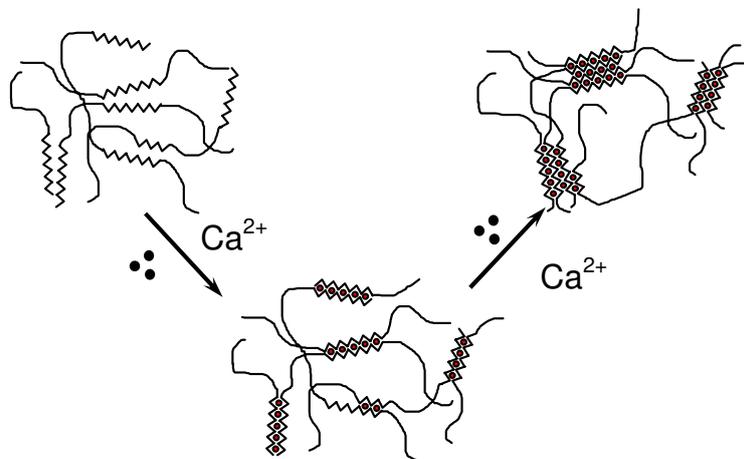


Fig. 1.6 Gelation mechanism of alginate in the presence of the divalent cation Ca^{2+} [27].

Alginate gels in the presence of some divalent cations (e.g. Ca^{2+} or Ba^{2+}), and the mechanism for gelation is widely accepted to be a creation of intermolecular G-block junction zones with egg-box structure (Fig. 1.6) [27, 28]. Alginate is widely studied and its gelling properties are highly exploited. It is a practical encapsulating agent and used in medical applications. In addition, its viscofying properties are also widely exploited in many food applications.

Alginate is not widely used as encapsulating agent in marine feed applications. It has however, been used to produce Gellyfeed (Rubin feed) [29]. In this application, by-products of the marine industry is mixed with alginate and CaCO_3 , and the mixture is extruded into an acid bath containing 5 % formic acid, where the pellets are left for 30 seconds to 12 hours to harden.

The encapsulation procedure of *C. finmarchicus* in this thesis is partly based on the gellyfeed method. However, target size (100-400 µm) of the particles was substantially smaller than those feed pellets produced by the traditional gellyfeed method (15 mm).

1.7. Fish gelatin (FG)

Fish gelatin (FG) is, as mammalian gelatin, a biomacromolecule derived from the fibrous protein collagen, which is a main constituent of connective tissue [30-33]. Sources of gelatin have traditionally been mammalian species such as bovine and pork, and the worldwide production in 2006 was 315,000 tons (www.gelatine.org). However, there has been an increasing interest for non-mammalian alternatives to gelatin, both from religious and health perspectives. Thus, sources such as Atlantic Cod (*Gadus Morhua*), Pollock (*Pollachius virens*) and Haddock (*Melanogrammus aeglefinus*) have been used to manufacture FG for the last three decades and the chemical and physical properties has been increasingly studied the last decades [34-36].

The main difference between these gelatins has been identified to be the content of glycine, proline and hydroxyproline, which is known to be correlated with the gelling, gel strength and melting temperature. Cold-water FG has a low content of pyrrolidine-rich regions and exhibit low gelling and melting points, 4-5 °C and 12-13 °C, respectively [31], whereas mammalian gelatins have higher gelling and melting points, 22-24 °C and 31-33 °C, respectively and are known to have a higher content of pyrrolidine-rich regions.

Gelatin gels are obtained by cooling gelatin solutions below the gelling temperature. The gel structure is the result of junction zone formation, where pyrrolidine-rich regions act as nucleation sites for formation of junction zones [37]. The length of these zones is probably composed of 20-30 amino acids, and it is hypothesized that the junction zones are stabilized by hydrogen bonds similar to those in native collagen.

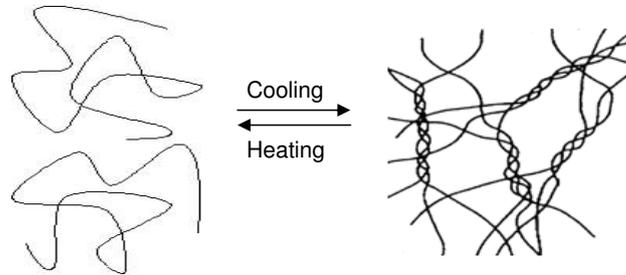


Fig. 1.7 FG as random coil α -chains and as a gel network.

Proteases are known to attack and degrade gelatin in solution where the gelatin molecules are present as random coils. The proteolytic degradation of gelatin is however decelerated or inhibited in gelatin gels where parts of the molecule exist in a triple helical structure [38].

The use of FG as an encapsulating agent may be of interest in e.g. fish feed formulations since its low melting point can facilitate the digestion in the fish and in addition act as a protein and energy source. Mechanical stability is, however, a crucial parameter in such an application. This is particularly important when using protease-containing zooplankton such as *C. finmarchicus* as a basic feed constituent.

Dynamic rheological methods are useful for studying the properties of viscous solutions and gels [36, 39]. An often used parameter for the elastic properties of viscoelastic gels is the dynamic storage modulus, G' , which is mathematically defined as:

$$G' = \left(\frac{\sigma_0}{\gamma_0} \right) \cdot \sin(\delta) \quad (2)$$

Where σ is the stress, γ is the amplitude of the strain and δ is the phase angle. The phase angle gives the offset between the stress and the strain curves. For perfect elastic materials the stress and the strain are in-phase ($\delta=0^\circ$). The phase angle is out of phase ($\delta=90^\circ$) for perfect viscous solutions. However, all other and real materials have a phase angle between 0° and 90° .

1.8. Chitosan

Chitosan is a marine polysaccharide derived from chitin, which is the structural component of the outer skeleton of crustaceans and insects [40]. Chitosans can be

considered as 2-deoxy 2-amino derivate of cellulose (Fig. 1.8). The precursor of chitosan, chitin, has mainly acetylated amino groups, whereas chitosan has between 100 and 60 % of the deacetylated form. Commercially, chitosans are produced by alkaline deacetylation of chitin to F_A values below 0.2-0.3 [40].

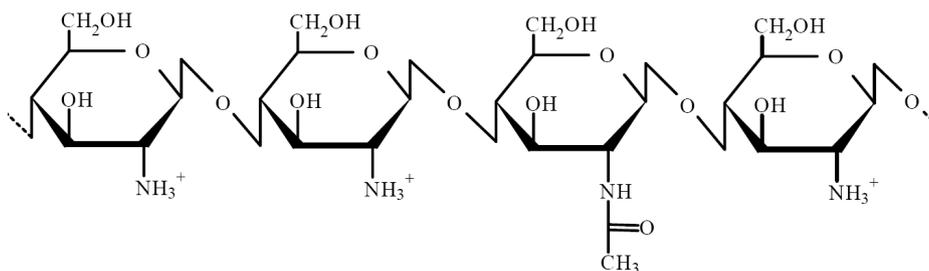


Fig. 1.8 Chemical structure of chitosan.

Chitosan is used in several applications (medical, health food etc.). Chitosans are positively charged at pH below the pK_A of the primary amino group (~6.5), and are used to coat negatively charged alginate capsules [41]. In this thesis, chitosans with different F_A were used to coat alginate capsules in order to control the release of the model molecule blue dextran. In addition, the stability in the presence of lysozyme was investigated since this is an enzyme, which could degrade the chitosan coating.

The glycosidic linkage $\beta(1\rightarrow4)$ in chitosan are hydrolyzed by chitinases, chitosanases and lysozyme. Lysozyme is an enzyme (EC 3.2.1.17) that degrades bacterial cell walls by hydrolysis of 1,4- β -linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan and between N-acetyl-D-glucosamine residues in chitin and chitosan [40]. It is abundant in a number of secretions, such as tears, saliva and mucus, and is also found in hen egg white (HEW).

The hydrolytic activity and the mechanism of lysozyme has been extensively studied. E.g., the activity of lysozyme is known to be highly affected by F_A (degree of acetylation), and experiments have shown that the rate of lysozyme hydrolysis is proportional with F_A^4 [41]. In addition, NMR studies have shown that lysozyme have an active binding site consisting of six subsites, commonly denoted A-F, where scission occurs between subsite D and E. Degradation studies and NMR determination of the identities have suggested that 4 N-acetylated residues have to be contained in the

lysozyme binding site to obtain maximum initial degradation rate. However, the binding of lysozyme to partially and highly N-actylated chitosans have been studied and it has been found that N-acetylated units (monomer) surrounded by deacetylated units bind preferentially in subsite C, without depolymerization of the chitosan chain [42, 43].

2. Experimental aspects

The materials and methods are described in the papers and the reader is referred to the appendix of this thesis for more details. However, some additional information about the raw material is given below in 2.1. In addition, materials and methods for unpublished results are described in 2.2.

2.1. The raw material

The raw material used in this thesis was caught in Norway, in the Trondheim fjord, in the sea outside the islands Hitra and Frøya. In addition, *C. finmarchicus* caught outside Bergen and Lofoten was used for comparison on biochemical composition in Paper 1. The *Calanus* was caught in net by trawling and was exposed to different sets of treatments. The main bulk was immediately frozen on dry ice, other parts were either treated with KOH, heat treated or left untreated until the entire catch was frozen at -40 °C at the Department of Biotechnology at NTNU in Trondheim.

Samples of the *Calanus* were taken out of the freezer and half-thawed at room temperature prior to experiments. The main preparation of the calanus was the production of water extracts for enzyme characterization and purification, but some parts were used for determination of biochemical composition, where main components were proteins, chitin, lipids and ash (minerals). Different kinds of protocols were used to extract and measure these components, and the methods are described in Paper 1. Homogenization of the raw material was not included in the extraction procedure. The batches of *C. finmarchicus* were significantly crushed during harvest, and further destruction of the material did not improve the extraction yield of protein. The high content of lipid (including phospholipids) and wax esters created difficulties in the extraction procedure, when homogenization was performed, since these molecules were probably emulsified in the extract. The exception was Paper 2 where homogenized *C. finmarchicus* was used in the alginate encapsulation procedure in order to produce homogenous *Calanus* model particles.

2.2. Analytical methods for unpublished results

2.2.1. Measurements of trypsin-like activity

The general proteolytic activity was performed as described in Paper 1. Trypsin-like activity was measured with a colorimetric assay with BAPNA as substrate [44], and was performed as follows: 43.5 mg of BAPNA (N α -Benzoyl-DL-arginine 4-nitroanilide hydrochloride) was dissolved in 1 ml DMSO. The volume was adjusted to 100 ml with 50 mM Tris-HCl pH 7.5. 0.9 ml 0.1 mM BAPNA was pipetted into a micro cuvette. 0.1 ml sample solution was added, and the increase in absorbance at 410 nm was recorded for 30 min. The initial linear region was used to calculate the activity using linear regression. This procedure was used in the protein purification steps to track trypsin-like activity.

The trypsin-like activity was measured for temperatures between 4 and 60 °C. In these cases, 0.9 ml substrate solution was mixed with 0.1 ml crude extract and incubated at 4, 10, 15, 20, 25, 30, 35, 40, 45, 50 or 60 °C. The reaction was arrested after 15 min with 1 ml 30% HAc, and the absorbance at 410 nm was measured. The molar coefficient 8800 M⁻¹ cm⁻¹ [45] was used to calculate the activity into the enzyme unit, U. One U equals 1 μ mol p-NA liberated per min. The activation energy was calculated using equation 2 as described in 1.4.1.

2.2.2. Chromatography methods

Crude extracts of *C. finmarchicus* were used as basis for protein purification. The extracts were defatted prior to purification with ice-cold tetrachloromethane (CCl₄) as described by Osnes and Mohr [46].

The defatted crude extract was subjected to anion exchange chromatography on a column packed with Toyopearl® DEAE-650C. Samples of 2 ml were applied to the column in 0.1 M Tris-HCl pH 7.5. The proteins were eluted at the same pH using a sodium chloride gradient (0 – 1 M) at a flow rate of 0.5 ml/h. 2 ml fractions were collected and analyzed for general proteolytic activity and protein concentration using the Biorad protein assay [47].

The defatted crude extracts were also subjected gel filtration using a spharose column in 0.1 M Tris-HCl, pH 7.5 at a flow rate of 0.5 ml/min. 2 ml fractions were collected and

analyzed for trypsin-like activity. In this case, the absorbance at 280 nm was measured to monitor the elution of proteins.

3. Results and general discussion

Section 3.1 deals with degradation of protein in *C. finmarchicus* and is based on Paper 1 and some unpublished results. Section 3.2 describes the release of proteinous components from encapsulated *C. finmarchicus* (Paper 2), whereas section 3.3 investigates the use of FG as a potential encapsulation agent for feed particle based on fresh *C. finmarchicus*. Finally, section 3.4 investigates the use of chitosan as a coating agent and the effect of the degree F_A on the release of a model substance.

3.1. Protease activity in *C. finmarchicus*

3.1.1. The biochemical composition of *C. finmarchicus*

The biochemical composition of some batches of *C. finmarchicus* from Norway (Trondheim, Bergen and Lofoten) was studied, and the major components were protein and amino acids, lipids including wax esters, and chitin (Paper 1). The results showed that *C. finmarchicus* contained, based on dry weight, 37-44 % protein-bound and free amino acids, 16-34 % lipid, 3-4 % chitin and 11-23 % ash.

The precise estimation of protein and free amino acids was difficult due to the proteolytic activity in the batches *post mortem*, and some part of the degraded protein may have been lost during storage on deck due to draining. However, it was estimated that about 16 % of total protein was water-soluble protein and 24 % exists as free amino acids, and the remaining 60 % were insoluble proteins. Hence, the majority of the amino acids were incorporated into water-insoluble protein. Water-soluble proteins are quickly degraded and lots of protein would be subsequently lost if suspended in water, which has been observed by Grabner [48]. The water-soluble fraction of protein in *C. finmarchicus* reported by Tonheim *et al.* [49] was higher (54 %) than described above, which may be due to a lower loss of water-soluble protein during the time between capture and freezing. This suggests that the handling of the catch is very important in order to get accurate estimation of the protein content in *C. finmarchicus*.

The variation in lipid content can be a result of differences in copepod stadia [50], i.e. the batches from Bergen and Trondheim may have had larger populations of stadium IV and V with respect to the Lofoten batch and hence a higher content of wax esters.

The ash content in the Lofoten batch was particularly high and a large proportion may be seawater ions. Differences in harvest methods and handling, i.e., draining time could explain this.

3.1.2. Proteolytic activity in *C. finmarchicus*

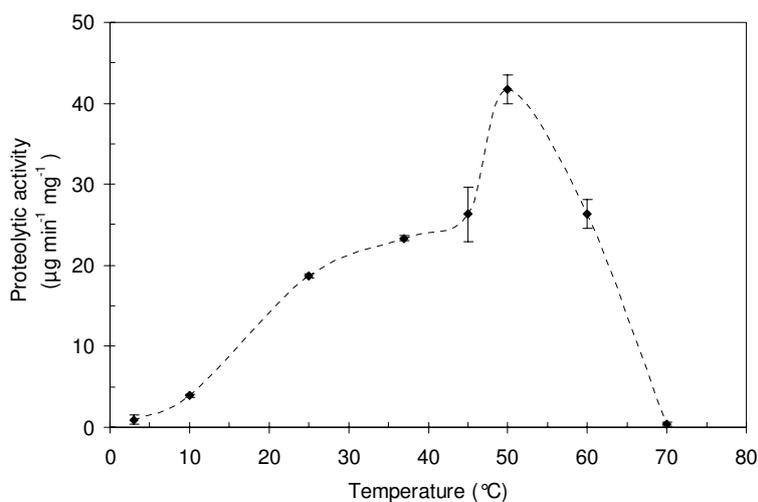


Fig. 3.1 General proteolytic activity in crude extract as function of temperature. Hemoglobin was incubated with crude extract for 3 h at pH 7. Assay mixture (2 ml) contained 0.2 % hemoglobin (v/w). The buffer used was 25 mM Mops pH 7. The error bars represent SDM.

The effect of temperature on the general proteolytic activity in crude extracts of *C. finmarchicus* was investigated. Fig. 3.1 shows that the proteases are most active between 10 and 60 $^{\circ}\text{C}$, with an optimum activity at 50 $^{\circ}\text{C}$. The result suggests, as expected, that a low temperature (below 10 $^{\circ}\text{C}$) is required to reduce proteolytic activity significantly. In addition, a high temperature (above 70 $^{\circ}\text{C}$) would be needed to stop the activity. Heat treatment between 70 and 100 $^{\circ}\text{C}$ should be sufficient to denature the proteases irreversibly, and may be used as treatment after harvest to stabilize the protein content in the batch. However, the rapid heating should be applied to avoid major degradation in temperature transition through the temperature optimum at 50 $^{\circ}\text{C}$.

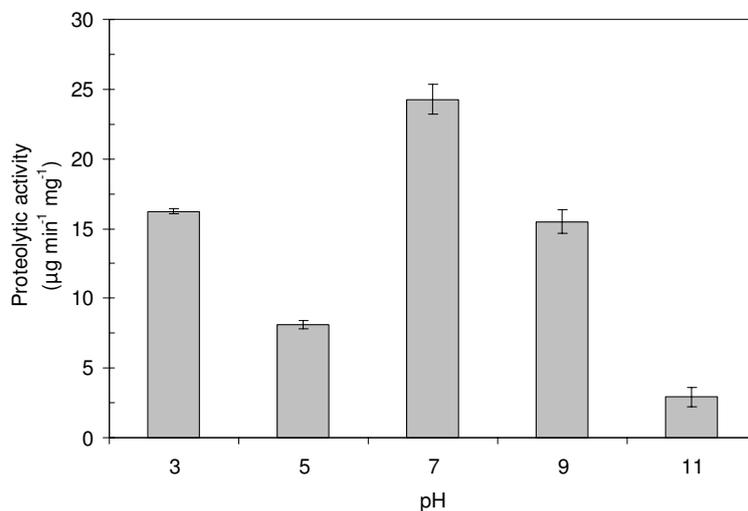


Fig. 3.2 General proteolytic activity in crude extract as a function of pH. Assay mixtures were incubated at 36 °C for 3 h. The error bars represent SDM.

Fig. 3.2 shows the general proteolytic activity as function of pH. The highest activity for the overall proteolytic activity was observed at neutral pH. The activity was found to be lowest at pH 11, as expected. The proteases are probably prone to irreversible denaturation and deamidation at this pH. This suggests that alkaline treatment may be a useful way to stabilize the protein content in the *C. finmarchicus* after harvest without the use of heat. Such treatment could be achieved by adding lye (NaOH or KOH) immediately and directly to the landed batch of *C. finmarchicus*. This could be a more economically way of stabilizing the protein as opposed to heat treatment which would demand much energy. However, some type of electric stirring device would be needed in order to mix the lye efficiently into large batches and to minimize the personnel's exposure to the strong lye. Alkali and heat treatment was performed on board a harvesting vessel in May 2006, and the effects of these treatments were studied and are presented in section 3.2 and Paper 2.

3.1.3. Protease classes in *C. finmarchicus*

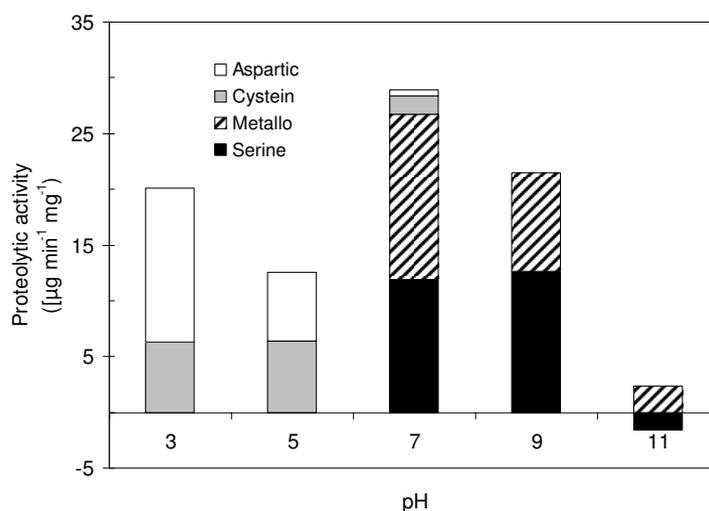


Fig. 3.3 Relative amounts of protease classes as function of pH.

The different classes of protease classes in crude extract of *C. finmarchicus* were identified and their relative activities are shown in Fig. 3.3 for different pH. The serine and metallo proteases were found to be dominant at neutral and alkaline pH, whereas aspartic and cysteine proteases were dominant at acid pH. This points toward possible inhibition methods at different pH. Addition of specific inhibitors against metallo and serine protease activity would be reasonable at neutral and alkaline conditions, and specific inhibitors against aspartic and cysteine protease activity at acidic conditions. These considerations may be useful in purifications of proteins and enzyme from *C. finmarchicus*, since it is important to avoid degradation of the target protein through the purification steps. Addition of inhibitors may be useful for stabilizing the protein content in *C. finmarchicus*. However, this may not be feasible in food and feed applications since inhibitors may prevent digestion or even be toxic. Some natural occurring protease inhibitors (e.g. inhibitors from legumes), may however, be considered used in low quantities to at least reduce protein degradation and this may be considered non-toxic.

3.1.4. Trypsin-like activity in *C. finmarchicus*

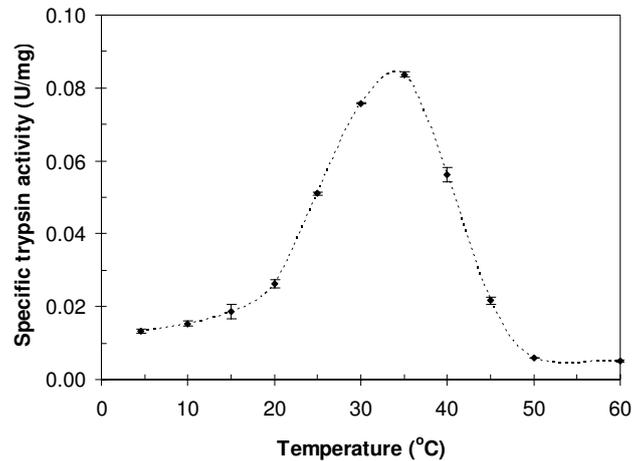


Fig. 3.4 Trypsin activity in crude *Calanus* extract as function of temperature. Buffer was 50 mM Tris-HCl, pH 7.5. Substrate was BAPNA. The error bars represent SDM.

Fig. 3.4 shows the trypsin-like activity in crude extract. The optimum temperature was 35 °C, which is significantly lower than the optimum temperature for the general proteolytic activity shown in Fig. 3.1, and is also lower than the temperature optima for trypsin-like activity in Antarctic Krill reported by Osnes *et al.* [51]. This suggests that other serine proteases and metallo proteases contribute to the activity above 35 °C in Fig. 3.1. In addition, the result in Fig. 3.4 suggests a heat treatment above 50 °C over some length of time would be sufficient to deactivate the trypsin-like activity in *C. finmarchicus*.

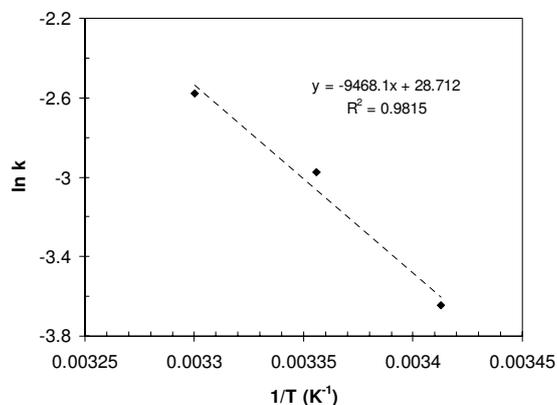


Fig. 3.5 Arrhenius plot for trypsin-like activity in crude extract of *C. finmarchicus*.

Fig. 3.5 shows the Arrhenius plot for the trypsin-like activity in *Calanus* extract in the temperature range 20-30 °C. The activation energy was calculated using the Arrhenius equation (equation 2., section 1.4.1), and was found to be approximately 79 kJ/mol, which is considerably higher than those found for trypsin-like enzymes in Antarctic Krill [51]. However, a different substrate (*p*-tosyl L-arginine methyl ester or TAME) and slightly different pH (8.1) was used for the calculation of E_A for Krill trypsins, which may have contributed to this difference. Moreover, the hydrolysis of TAME was reported to be significantly faster than the hydrolysis of BAPNA, suggesting that the hydrolytic properties of trypsins are highly substrate dependent. However, the properties of proteolytic enzymes in *C. finmarchicus* are not widely known and further studies are needed.

3.1.5. Partial purification of proteases in the *C. finmarchicus*

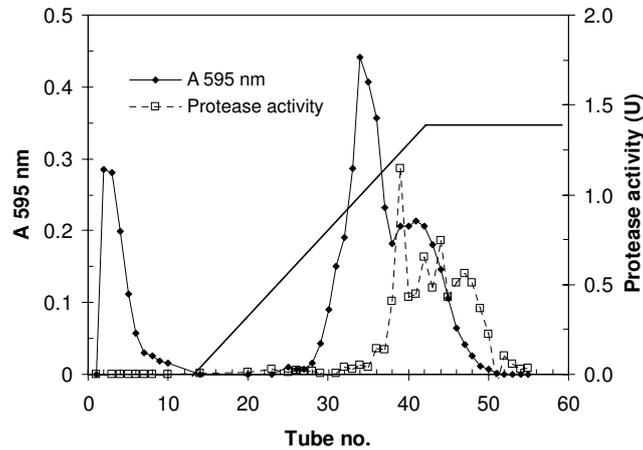


Fig. 3.6 Purification of proteases from *C. finmarchicus* on anionic exchange column. Proteins were eluted with a gradient of NaCl (0-1.0 M) in 100 mM Tris-HCl, pH 7.5, at a flow rate of 0.5 ml/min. General proteolytic activity was measured at pH 7 and 37 °C.

Anionic chromatography should be a useful step for the purification of proteases from *C. finmarchicus* (Fig. 3.6). Cationic proteins were eluted in the first peak, whereas the anionic proteins were eluted as the salt concentration increased above approximately 0.5 M. Two peaks with anionic proteins were eluted close to each other with no baseline separation, which may have been improved by using a different salt gradient setup. Protease activity was only measured in the third peak, and the data suggests that this peak may contain several anionic proteases as has been detected in Krill [46, 51, 52].

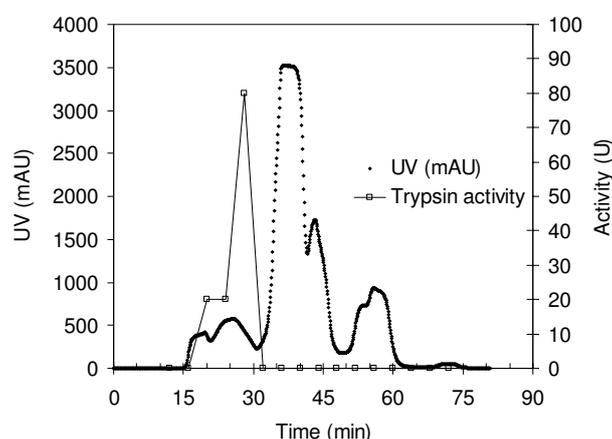


Fig. 3.7 Gel filtration on a spharose column. Proteins were eluted with 100 mM Tris-HCl, pH 7.5, at a flow rate of 0.5 ml/min.

A different approach to protease purification was gel filtration. Fig. 3.7 shows the elution profile for defatted crude calanus extract. Proteins with trypsin-like activity were eluted among the first proteins. These fractions were collected and subjected to affinity chromatography. The affinity material bound the trypsin-like enzymes; however, those enzymes were not successfully eluted from the affinity material. Further purification attempts were not performed due to time limitations.

A purification scheme for proteases may be suggested based on the partial results shown in this thesis. Trypsin-like enzymes could be purified by applying anion-exchange-, size-exclusion-, and affinity chromatography, and to perform these at constant low temperature (e.g. 4 °C) to keep the proteolysis on a minimum. An additional strategy would be to add protease inhibitors, which are not specific to trypsin-like enzymes. At neutral and alkaline pH, inhibitor towards chymotrypsin and especially metallo proteases could be useful. A purified protease should be characterized based on molecular weight, pH and temperature optima and isoelectric point. In addition, the primary structure could be sequenced, and the amino acid sequence (from N-terminal) could be compared with protease from other sources (a phylogenetic study).

3.2. Release of proteinous components from alginate-*Calanus* particles

Section 3.1 and Paper 1 indicate that the protein content of *C. finmarchicus* is rapidly degraded into oligopeptides and free amino acids. Thus, it was decided to study the release of these components from Ca-alginate encapsulated *C. finmarchicus* and the details of this study were presented in Paper 2.

The main purpose with this study was to investigate the effect of parameters such as pH, ionic strength and temperature on release. These parameters were adjusted far away from physiological conditions in order to study the importance of electrostatic interactions between the poly-anion alginate and protein. The ninhydrin method was used in some of the experiments to get a rough picture of the release of small proteinous components. A more detailed study on the release of specific components could be achieved by using e.g. HPLC chromatography, which would have shown release of specific free amino acids, however, it was decided that this would be unnecessarily detailed in order to investigate the overall effects of pH, ionic strength and temperature.

3.2.1. Release of amino groups

Figure 3.8 shows the increase in the number of amino groups in the incubation buffer at different temperatures as measured by the ninhydrin method. Ninhydrin solution reacts primarily with primary amino groups on proteins and amino acids but can also react with ammonia and with other amino groups on proteins and other molecules [22]. Hence, the term “amino groups” is used in this study to describe all components reacting with ninhydrin. However, protein hydrolysis will inevitably create more ninhydrin reactive primary amino groups implying that the colorimetric response will increase with increasing protein degradation. It can thus be assumed that the data in Fig. 3.8 primarily reflects the release of free amino acids and oligopeptides.

The release increased with temperature, as expected, probably due to increased proteolytic activity, as the products of proteolysis (amino acids, short oligopeptides) would easily diffuse out of the alginate network.

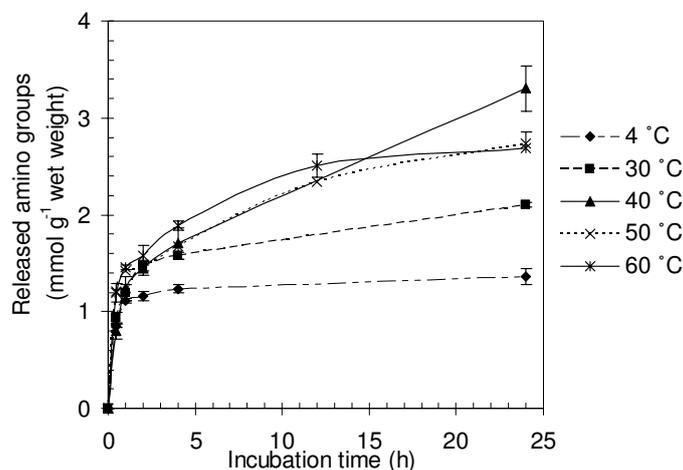


Fig. 3.8 Release of amino groups as a function of time at five different temperatures. Buffer was 50 mM Mops, pH 7. The error bars represent SDM.

At all temperatures, there was initially a high release of amino groups from the encapsulated material within the first two hours of incubation. This initial increase can be attributed to the release of free amino acids, which are present in zooplankton as osmoregulators [53] and from predigested protein before the catch was frozen. This release of small components was probably only limited by the diffusion rate. Small molecules are known to diffuse very rapidly within an alginate network. Martinsen *et al.* [54] found that a molecular weight of more than 300 kDa was needed for globular protein molecules to exhibit diffusion constants lower than that of pure water. However, the particles will also contain active proteases, which over time will degrade the water soluble proteins and on a later stage probably also the non-water soluble proteins from the *C. finmarchicus* (Paper 1). The latter process was significantly slower and the release after 2 hours can thus be regarded as limited by this proteolytic action.

The rate of release was significantly lower after two hours for all temperatures; however, a clear temperature dependency was observed. The release rate increases with temperature up to 40 °C. At 50 and 60 °C, a reduced rate was observed after approximately 12 hours of incubation, probably reflecting protease instability (denaturation) at these relative high temperatures (Paper 1). As expected, the lowest rate

of release was at 4 °C since the proteolytic activity here is known to be low (Paper 1). This result shows that thawed *Calanus* as well as fresh feed particles should be stored at low temperatures (below 4 °C) to avoid an unacceptable loss of protein.

It is likely that both exo- and endo proteases were active in the model feed particles. However, it would be reasonable to suggest that the exo-proteases were more responsible for the accumulation of free amino acids in the system since they release free amino acids, tri- and dipeptides from the terminal ends of the proteins. However, the endo-proteases were probably involved indirectly since they increase the amount of accessible substrate for the exo-proteases.

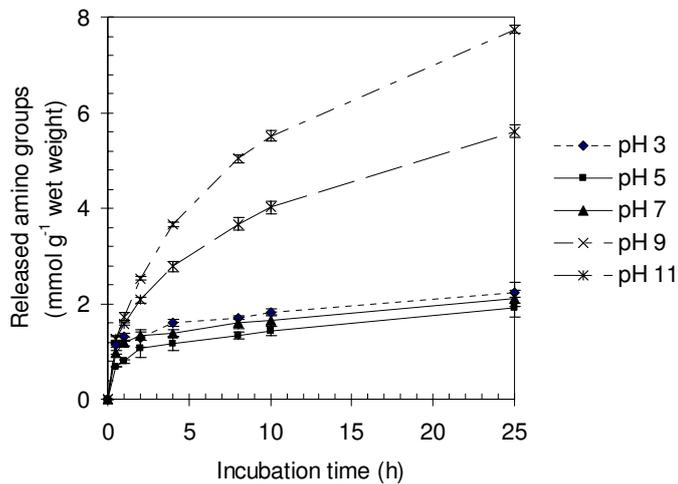


Fig. 3.9 Released amino groups as a function of time and pH. The incubation temperature was 40 °C. The error bars represent SDM.

Fig. 3.9 shows the effect of pH on released amino groups. The release was highest at pH 9 and 11, which would suggest that an elevated pH while producing feed capsules based on alginate and *C. finmarchicus* could lead to significant loss of protein. The high release at pH 9 may be due to increased exo-activity at this pH compared to pH 7, whereas the apparent high release at pH 11 was probably due to deamidation and release of NH₃ and not due to proteolytic activity, as shown in Paper 1.

3.2.2. Release of protein

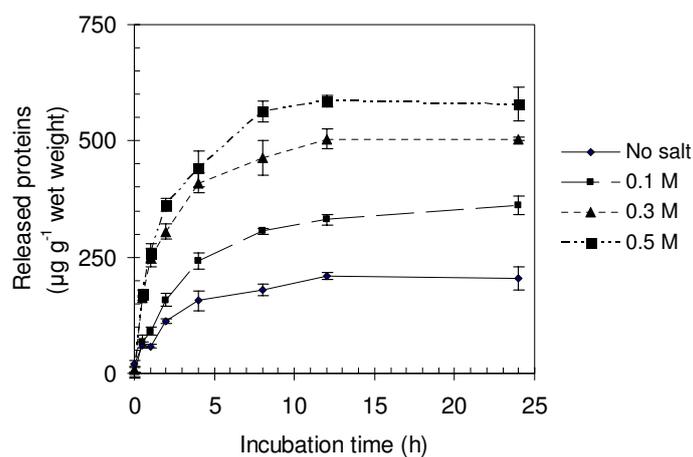


Fig. 3.10 Release of proteins (>3 kDa) as function of incubation time and NaCl concentration. Incubation temperature was 40 °C and the incubation medium was 50 mM acetate, pH 5. The error bars represent SDM.

The effect of ionic strength on protein release at pH 5 is shown in Fig. 3.10. The release of protein was clearly affected by ionic strength, and the data suggest that increased ionic strength led to increased release. The electrostatic interactions between poly-anion alginate and protein may have been affected by the increased ionic strength. At pH 5, the carboxyl group will carry a negative charge ($pK_A \sim 3.5$), which suggest that electrostatic attractive interaction may take place between the alginate network and positively charged patches on the water-soluble proteins at low ionic strengths. The effect of added salt shown in Fig. 3.10 may have been due to shielding off of short range attractive forces [55] as well as reducing the entropic driving force of the release of condensed counterions, and hence an increased release of proteins from the capsules with increasing ionic strengths.

In addition to the above-mentioned effects, there may have been an increased solubility of some proteins (salt soluble proteins) at higher ionic strengths (“salting in” effect). This effect is known for proteins containing a low incidence of non-polar patches

[56]. Hence, higher solubility of salt soluble protein could be partly responsible for the increased release at higher ionic strength shown in Fig. 3.10.

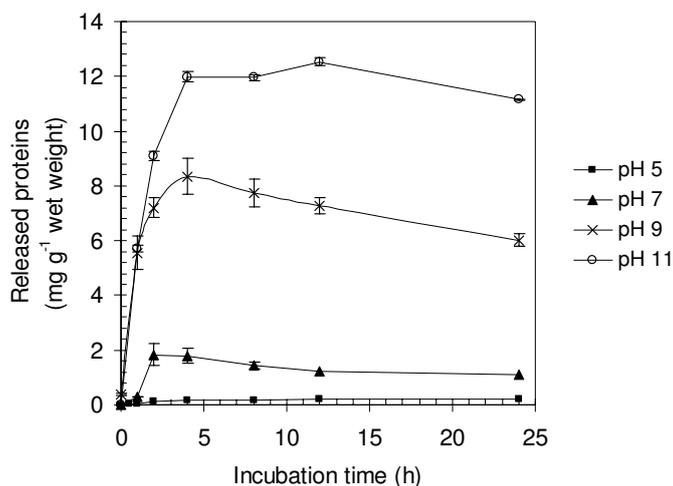


Fig. 3.11 Release of proteins (> 3 kDa) as a function of time and pH. Incubation temperature was 40 °C. The error bars represent SDM.

Fig. 3.9 and 3.11 show a high degree of release at pH 9 and 11 compared to pH 7. This may partly be due to an increased solubility of protein at higher pH [57]. The data in Fig. 3.9 suggest that the highest degree of release was at pH 9 whereas the data in Fig. 3.11 apparently show more release at pH 11. However, it is likely that there is also a co-release of proteolytic enzymes into the surrounding medium, which subsequently would degrade released proteins into small peptides and amino acids efficiently at pH 9. Thus, Fig. 3.11 reflects both release of proteins from alginate encapsulated *C. finmarchicus* at pH 9 and 11 as well as proteolytic activity in the surrounding medium at pH 9 leading to low molecular weight proteinous material not detectable with the Bio-Rad method [47]. Moreover, the apparent reduction in released proteins with time at pH 9 (Fig. 3.11) supports the view that such proteolytic degradation took place.

The increased release at pH 9 and 11 (Fig. 3.11) was most likely due to electrostatic repulsion between the negatively charged alginate network and proteins carrying a net negative charge at high pH. This may also explain why there was a considerably lower release at neutral and acidic conditions.

3.2.3. Effect of pretreatment on release of protein and amino groups

In order to exam the possibility of controlling the release of proteinous material from model particles two different preservation methods were compared with encapsulated fresh material (Fig. 3.12 and 3.13).

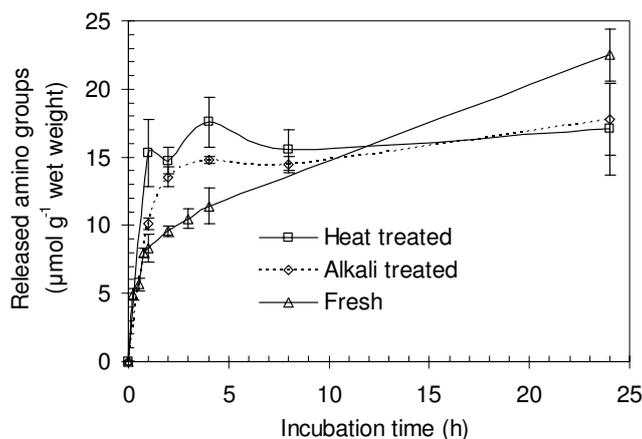


Fig. 3.12 Release of amino groups from feed particles made of heat treated, fresh and alkali treated *C. finmarchicus*. The incubation medium was 50 mM Mops, pH 7. Incubation temperature was 40 °C. The error bars represent SDM.

Figure 3.12 shows the release of amino groups from models feed particles made from heat-treated, alkali treated and fresh *C. finmarchicus*. The level of free amino groups in the heat-treated sample was around 18-19 µmol per g wet weight after only one hour of incubation, suggesting that there was a high initial content of free amino acids in the heat-treated *C. finmarchicus*. Since no further release was observed, it is reasonable to anticipate that the proteases were inactivated. The high release of amino groups from the heat-treated *C. finmarchicus* was partly due to the presence of osmoregulating amino acids, but also caused by an accelerated protease activity early in the heating process. However, the data also suggest that most of the proteolytic activity was lost after treatment at 80 °C for 15 min. A rapid heating process would therefore be recommended to avoid loss of protein components during this preservation method.

The release of amino groups from feed particles of alkali treated *C. finmarchicus* was also high the first two hours, again reflecting the inherent content of free amino acids, but also suggesting that there may have been some degree of protein hydrolysis in the alkali treated *C. finmarchicus*. In addition, the high pH (~11) may have altered some non-water soluble proteins into water-soluble molecules, and there may have been some release of NH_3 , which would interfere with the ninhydrin method.

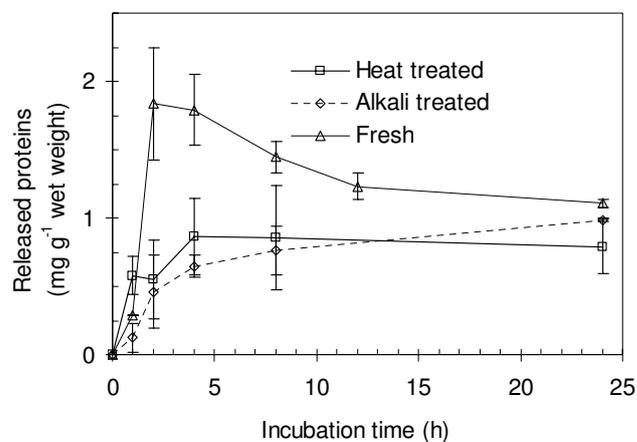


Fig. 3.13 Release of proteins (>3kda) from feed particles made of heat treated, alkali treated and fresh *C. finmarchicus*. The incubation medium was 50 mM Mops, pH 7. Incubation temperature was 40 °C. The error bars represent SDM.

Fig. 3.13 shows the release of proteins from feed particles made from heat treated, alkali treated and fresh (untreated) *C. finmarchicus*, showing a significant difference in the release of proteins between treated and untreated samples. Particles of fresh biomaterial released more than twice as much protein during the first two hours followed by a clear decrease in medium protein content after 4 hours, again suggesting proteolytic activity in the medium. The heat and alkali treated particles showed no significant decrease in medium protein, confirming that most of the protease activity was lost. In essence, Fig. 3.12 and 3.13 show that fresh model feed particles released more large protein entities and also an increasing concentration of free amino acids and oligopeptides over time due to intact proteolytic enzymes than do alkali and heat treated biological material.

3.3. Proteolytic degradation of FG with proteases from *C. finmarchicus*

FG is both a protein and gelling agent, and could serve as an encapsulation agent as well as a protein and energy source in marine feed as long as the temperature is kept below FG's melting temperature. However, the encapsulated material should not interact with the FG in any way that would weaken the mechanical strength. The fact that *C. finmarchicus* contains proteases suggests that FG would be proteolytically attacked if this species were encapsulated with FG. In this thesis, the stability of FG in solution and as gel in the presence of *Calanus* proteases was investigated, and the main results are discussed in 3.3.1-3.3.2. Other results and a more thorough discussion can be found in Paper 3.

3.3.1. Degradation of FG solution

Fig. 3.14 shows the degradation of FG in solution measured with capillary viscometry. This degradation indicates that FG functions as substrate for the proteases in *C. finmarchicus* when FG is in a sol state and at temperatures above 20 °C.

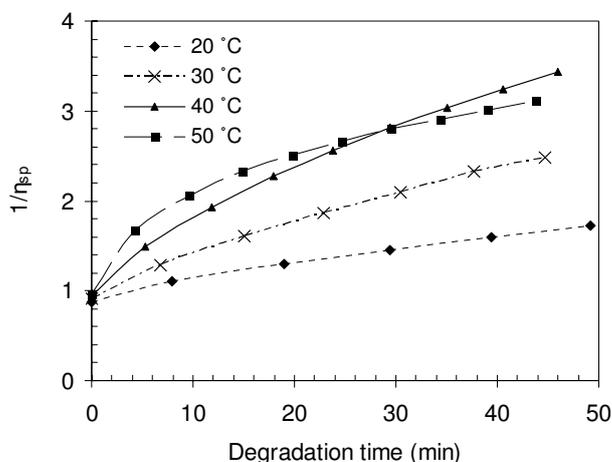


Fig. 3.14 Degradation of 1 % (w/v) FG (0.067 mg/ml *Calanus* protein, 50 mM Tris-HCl, pH 7.5) measured by capillary viscometry at 20, 30, 40 and 50 °C.

A depolymerization of FG by proteases was not unexpected since the FG molecules at these temperatures exist as random coils in solution, totally lacking the protective triple helical structure found in collagen [38]. Hence, the random coil α -chains were readily

exposed to proteolytic attacks causing a reduction in the average molecular weight and changing the molecular weight distribution. The reduced degree of polymerisation of the FG molecules led to a reduction in the specific viscosity. The decline in specific viscosity suggests that the crude extract contained endo-proteases, since internal proteolysis in the protein chains is required to give a depolymerization leading to a reduction in specific viscosity. Again, and as described also in Papers 1 and 2, the proteolytic activity was reduced at 50 °C indicating protease instability.

3.3.2. Proteolytic degradation of FG gels

Rheological methods were applied to study the effect of *Calanus* proteases on FG in the gel state and at low temperatures (below 12 °C). This was achieved by measuring the dynamic storage modulus (G') as function of time at curing temperatures between 4 and 10 °C. Room tempered solutions of 10 % FG was applied to the rheometer at 4 °C, which led to rapid cooling and gelation of the sample. The temperature was thereafter ramped to 6, 8 or 10 °C after 2 hours of curing at 4 °C (Fig. 3.15). The experiments were also performed in the presence of *Calanus* proteases (Fig. 3.16). The major results are briefly discussed below.

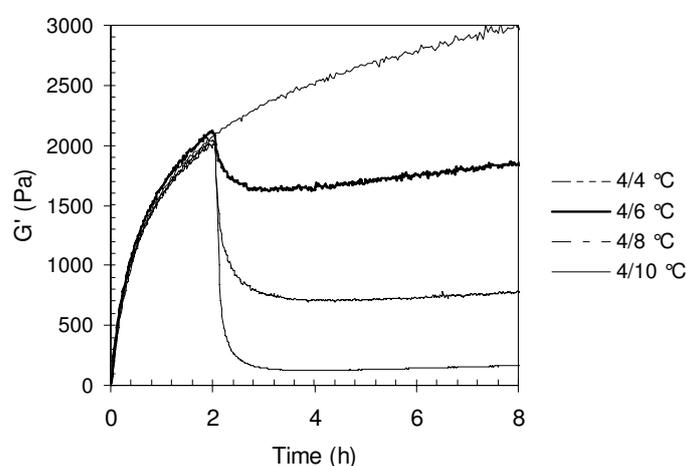


Fig. 3.15 Evolution of the dynamic storage modulus (G') upon time after curing at 4 °C (2 h) and after subsequent ramping to 6, 8 and 10 °C for 10 % (w/v) FG (50mM Tris-HCl, pH 7.5.).

The effect of proteases on G' is easily observed when comparing Fig. 3.15 and 3.16. The first two hours of curing showed an increase in G' in both cases, but the increase was considerably lower in the presence of proteases. Fig. 3.15 suggests that there was some gradual recovery of the gel strength with time after temperature ramping. In presence of proteases however, no such recovery was observed. In fact, G' started declining after two hours without temperature ramping (constant 4 °C), indicating hydrolysis of the FG molecules. This was most likely due to proteolytic cleavage within the elastic segments during curing at 4 °C. A slight depolymerisation may however, also have occurred as a result of proteolytic action during mixing at room temperature prior to the rheological analysis.

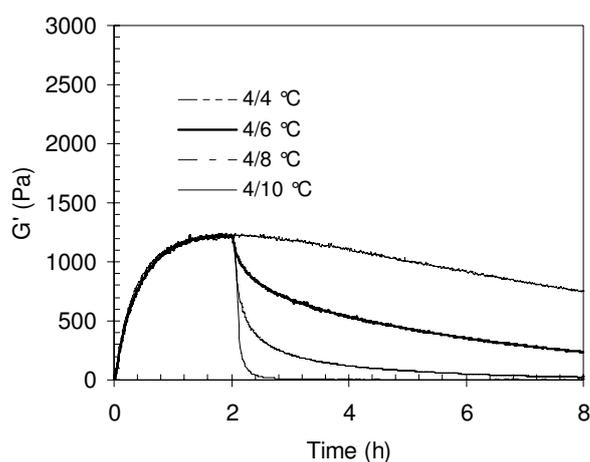


Fig. 3.16 Evolution of the dynamic storage modulus (G') upon time after curing at 4 °C (2 h) and after subsequent ramping to 6, 8 and 10 °C for 10 % (w/v) FG (50mM Tris-HCl, pH 7.5) in the presence of proteases (0.12 mg/ml *Calanus* crude protein).

It is reasonable to anticipate that interruption and damages, like e.g. minor depolymerisation of molecules, will influence the sensitive initial formation of a gelatin gel network and thereby also the mechanical properties of these networks as a function of time. This may explain that even though the activity of the *Calanus* proteases was low at 4 °C (Paper 1), it may be sufficient to cause a reduced elasticity.

On the basis on above results, it may be concluded that FG is not suitable as an encapsulated for fresh *C. finmarchicus* due to the proteolytic activity. The protease activity would significantly destabilize the mechanical strength in such feed particles. Hence, the *C. finmarchicus* would have to be processed to eliminate the protease activity prior to encapsulation.

3.4. Some release properties of chitosan coated alginate capsules

Positively charged chitosan molecules may bind to negatively charged surfaces, and it is known to be bioadhesive i.e. bind to negatively charged cell walls and other living tissues [40]. Chitosan coating may be used to control the release of nutrients from feed particles. In this thesis, the effects of F_A and the presence of lysozyme on the release of blue dextran (BD) from chitosan coated capsules were investigated. In addition, some initial results using hemoglobin as a model release substance are discussed. All capsules were produced by the one-stage method and the results are presented and discussed in 3.4.1 to 3.4.3.

3.4.1. Release of hemoglobin

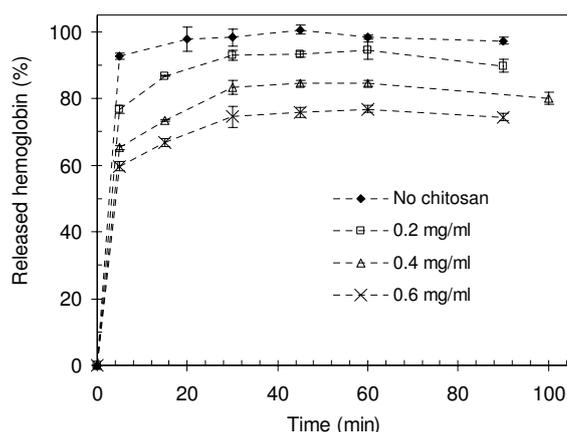


Fig. 3.17 Release of hemoglobin as function of time for different concentration of chitosan in the gelling bath ($F_A=0.12$). The buffer was 50 mM NaAc/100 mM CaCl₂, pH 5. The error bars represent SDM.

Fig. 3.17 shows the release of hemoglobin from chitosan coated alginate capsules. There was a rapid release the first minutes at all chitosan concentrations. In the case of no chitosan, almost all hemoglobin was released within the first half hour, which suggests that the hemoglobin molecules rapidly diffuse out of the alginate network. A similar high release of hemoglobin has been reported earlier [58]. The results showed also that the presence of chitosan had a significant effect on the release, and that the release decreased with increasing concentration of chitosan in the gelling bath.

Hemoglobin has an isoelectric point at 6.8, which means that the protein has a net positive charge at pH 5 and may have participated in the electrostatic bridging. However, the rapid and extensive release shown in Fig. 3.17 suggests that such interaction did not play a considerable role. The presence of chitosan seemed to decrease the release of hemoglobin probably due to coating thereby reducing the permeability of the capsules.

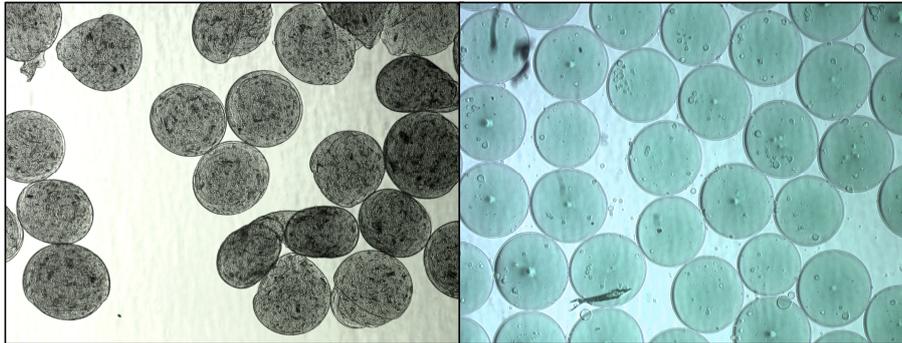


Fig. 3.18 Hemoglobin (left) and BD (right) encapsulated in Ca-alginate capsules with chitosan coating.

Fig. 3.18 (left) shows a picture of capsules with chitosan coated Ca-alginate with hemoglobin. Most of them were round, but approximately 30 % of them were misshaped including some, which were attached to each other (flocculation). It is uncertain what caused these misshaped capsules. It may have been the chitosan layer, which burst due to rapid release of hemoglobin during the encapsulation. At least, it seems that the presence of hemoglobin contributed to the effect since no misshaping was observed in the study with BD as seen in Fig. 3.18 (right).

3.4.2. Release of blue dextran

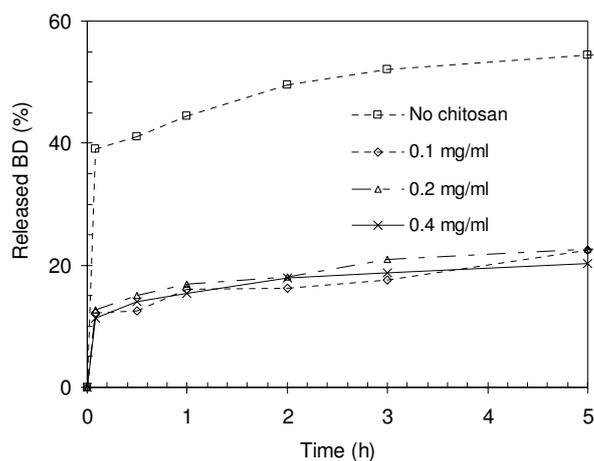


Fig. 3.19 Release of BD from chitosan coated alginate capsules as function of chitosan concentration in the gelling bath ($F_A=0.12$). Buffer was 50 mM NaAc/100 mM CaCl₂, pH 5. The error bars represent SDM.

Fig. 3.19 shows the release of BD from alginate capsules coated with chitosan with $F_A=0.12$. In the case of no coating, the initial release was significant (40 %) and it continued to increase with time, reaching approximately 54 % after 5 hours. The introduction of chitosan led to a significant reduction in BD release, which suggests that the capsules have been effectively coated and that diffusion of BD out of the alginate matrix has been obstructed by the presence of chitosan on the surface. In addition, there seemed to be no concentration dependence above 0.1 mg/ml chitosan in the gelling bath, which suggests that the surface of the alginate capsules has been saturated with chitosan already at 0.1 mg/ml chitosan. BD is a large biomolecule with an average molecular weight of approximately 2 million Da, and is expected to have a slow release from such particles. It would therefore be reasonable to suggest that only a small amount of chitosan would be needed to reduce the release of such large molecules.

High G-alginates create open networks, which allow fairly large molecules to diffuse easily out of such a network [54]. Even if BD has a significant higher molecular weight, it may still diffuse out due to its compact conformation. Dextrans have $\alpha(1\rightarrow6)$

glycosidic linkages which are very flexible. Hence, dextrans have less extended molecular conformation than most other polysaccharides [59].

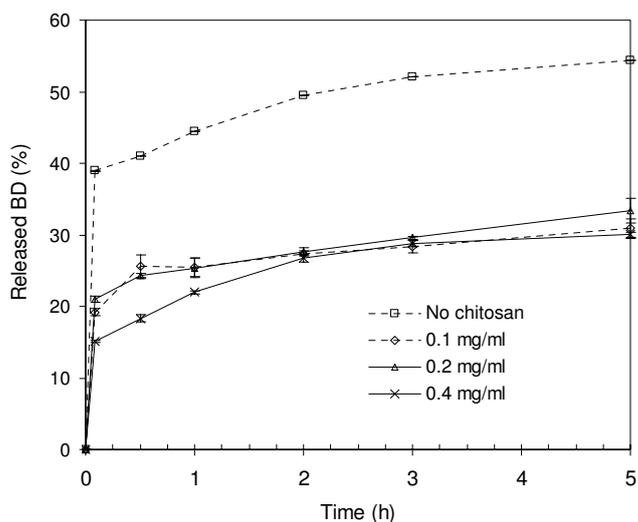


Fig. 3.20 Release of BD from chitosan coated alginate capsules as function of chitosan concentration in the gelling bath ($F_A=0.40$). Buffer was 50 mM NaAc/100 mM CaCl₂, pH 5. The error bars represent SDM.

Fig. 3.20 shows the release from capsules coated with chitosan with $F_A=0.4$ at three different concentrations. No significant difference was observed for 0.1 and 0.2 mg/ml chitosan. At 0.4 mg/ml however, there seemed to be a significant lower release the 2 first hours. After 2 hours, the release was similar to the two lower concentrations, suggesting that higher concentration of chitosan only affects the initial release of BD and does not have any effect on longer storage times.

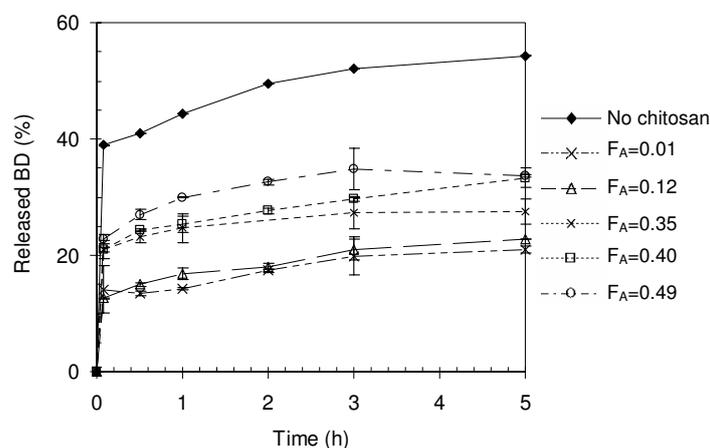


Fig. 3.21 Release of blue dextran as function of F_A . The buffer was 50 mM Acetate/100 mM CaCl_2 , pH 5. The chitosan concentration was 0.2 mg/ml. The error bars represent SDM.

Fig. 3.21 shows the release as function of F_A . The release was lowest for capsules coated with the least acetylated chitosan ($F_A = 0.01$), as expected. This chitosan coating led to a approximately 60 % reduction in the release of blue dextran after 5 hours. However, the release with $F_A=0.01$ was not significantly lower than that of capsules with chitosan with $F_A=0.12$, suggesting that a degree of acetylation of 0.12 is sufficient for effective coating. Chitosan with $F_A=0.4$ and 0.49 led only to approximately 35-40 % reduction in BD release after 5 hours. This release is significantly higher, which suggests that chitosans with higher degree of acetylation are less effective as coaters due to a lesser amount of positive charges on the chitosan molecule as well as carrying more bulky acetyl groups preventing effective molecular packing.

3.4.3. Effect of lysozyme on BD release

The effect of lysozyme on chitosan coated capsules was investigated for chitosans with F_A of 0.12 and 0.40. The concentration of chitosan in the gelling bath was kept at 0.2 mg/ml.

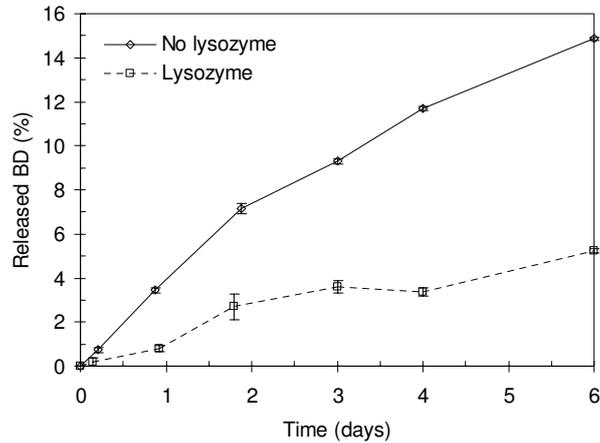


Fig. 3.22 Effect of HEW lysozyme on chitosan coated and washed alginate capsules containing BD ($F_A=0.12$). Buffer was 50 mM NaAc/10 mM CaCl_2 , pH 5. The lysozyme concentration was 0.6 mg/ml. The error bars represent SDM.

Fig. 3.22 shows the effect of lysozyme on coating with $F_A=0.12$. The release was significantly inhibited by the presence of lysozyme, which may seem surprising. However, lysozyme is known to attach strongly to chitosans with a high fraction of mono-acetylated monomers without subsequent hydrolysis. It is therefore reasonable to suspect that the release is reduced due to the formation of a lysozyme-chitosan layer on the surface of the alginate capsules. In addition, HEW lysozyme is a small, globular peptide (~14 kDa) which should be able to diffuse easily into the alginate capsule and saturate the network. At pH 5, the HEW lysozyme molecule has a strong net positive charge (pI 11.35), and should be able to bind to the negatively charged carboxyl groups on the alginate. All in all, these effects would obstruct the release of dextran leading to a reduced release.

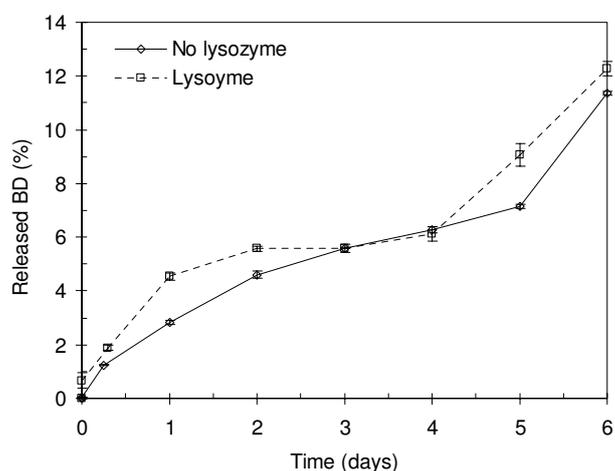


Fig. 3.23 Effect of HEW lysozyme on chitosan coated and washed alginate capsules containing BD ($F_A=0.4$). Buffer was 50 mM NaAc/10 mM CaCl₂, pH 5. The lysozyme concentration was 0.6 mg/ml. The error bars represent SDM.

Fig. 3.23 shows the effect of lysozyme for $F_A=0.4$. The presence of lysozyme led to an increased release the first 2 days, followed by a 2 day lag period before a new period of release on the following 3 last days of incubation. The release of BD seemed to be affected by lysozyme; however, the non-linear behavior was not as expected. This suggests that lysozyme is degrading the chitosan layer leading to an initial higher release of BD. As mentioned above, HEW lysozyme has a net positive charge and may therefore interact electrostatically with the alginate network, which again may have slowed the hydrolysis of chitosan and led to lysozyme saturated capsules. However, the detailed reason for this non-linear behavior is not yet fully understood, and further experiments are required.

By comparing Fig. 3.22 and Fig. 3.23, it can be seen that the total release of BD after 6 days are higher in the case with $F_A=0.12$ than $F_A=0.4$ in the absence of lysozyme. This was surprising; however, one possible explanation may be the difference in BD content in the capsules after washing. Capsules with $F_A=0.12$ contained 0.85-0.86 % BD, whereas capsules with $F_A=0.4$ contained 0.78-79 %. Thus, capsules coated with chitosan with $F_A=0.12$ would have had a slightly higher concentration gradient between the interior of

the capsules and the surrounding buffer. This driving force, though weak, may explain some of the difference between Fig. 3.22 and Fig. 3.23.

4. Concluding remarks and future perspectives

This thesis has focused on model feed particles made from zooplankton, and challenges of protein preservation and release of encapsulated protein and model molecules. The proteolytic activity in *C. finmarchicus* was studied in some detail; however, a more detailed study on the enzymes would be of interest. The different protease classes were identified, but no protease was purified and characterized. A purification strategy was attempted but the work was not completed during this thesis. The main obstacle was the high proteolytic activity in the raw material, which effectively degraded the target proteases (trypsin-like enzymes) during the purification steps.

Fresh wet *C. finmarchicus* is not likely the best choice as basic feed ingredient, since it is too unstable due to autolysis. In addition, batch variation could make it difficult to obtain stable feed quality. Treatments with heat and lye may reduce catabolism; however, the final content of protein, lipids and other nutrients would vary depending on season, copepod stadia and handling *post mortem*. Thus, the *C. finmarchicus* should be processed soon after catch with heat to minimize protein loss if it is to be used in wet form.

Paper 1 identified the protease classes in *C. finmarchicus* and this result suggests that protease inhibitors may be used to eliminate or reduce the proteolytic activity. One possibility could be to incorporate soy bean powder, which has a natural content of serine protease inhibitors. However, any such addition of inhibitors may also reduce the digestibility of the feed particles.

Paper 3 studied FG as a potential encapsulation agent, and protein and energy source in feed particles. The study showed however, that this molecule was readily degraded in the presence of *Calanus* proteases, again indicating that the protease activity in *C. finmarchicus* is a major challenge, even at temperature below 12 °C.

The results in Paper 2 showed that alginate encapsulated *C. finmarchicus* exhibited a significant release of proteinous components, especially at elevated pH and ionic strength. That study investigated effects of pH, temperature and ionic strength far from physiological conditions, and was intended to show release behavior under potential

processing conditions prior to feeding. Overall, the results in this paper points towards additional methods to be exploited to control the loss of protein.

A possible method to control the release of proteins and other nutrients from feed particles may be chitosan coating. However, the choice of chitosan would be of major importance. As suggest in section 3.4, the degree of acetylation is probably the most important parameter as it influences the chitosan's ability to attach to alginate surfaces and determines the stability in presence of e.g. lysozyme. A highly deacetylated chitosan would probably not be the appropriate choice since non-productive binding between chitosan and lysozyme may halt the digestion of such feed particle in e.g. the GI tract.

Finally, the initial thought of using alginate encapsulated *C. finmarchicus* as cod larval feed was encountered by significantly low protein stability in the raw material due to autolysis. Based on this thesis, it could be suggested, that the best approach would be to eliminate the protease activity by heat and subsequently dry the *C. finmarchicus* into a powder. Such a powder could be used as an additive in the larvae feed provided that the protein digestibility is preserved.

List of symbols and abbreviations

BAPNA	N α -Benzoyl-DL-arginine 4-nitroanilide hydrochloride
BD	Blue dextran
DHA	Docosahexapentaenoic acid
DPH	Days post hatching
E _A	Activation energy (J/mol)
EPA	Eicosapentaenoic acid
F _A	Degree of acetylation
FG	Fish gelatin
G'	Dynamic storage modulus (Pa)
HEW	Hen egg white
HUFA	Highly unsaturated fatty acid
M _w	Weight average molecular weight (g/mol, Da)
SDM	Standard deviation of mean
TAME	<i>p</i> -tosyl L-arginine methyl ester
η_{sp}	Specific viscosity (ml/g)

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Paper I



Proteolytic activity and protease classes in the zooplankton species *Calanus finmarchicus*

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Received 9 November 2006; received in revised form 27 February 2007; accepted 27 February 2007
Available online 6 March 2007

Abstract

The temperature optimum for the general proteolytic activity in the crude extract of *Calanus finmarchicus* was 50 °C and the pH optimum was found to be 7. The use of specific protease inhibitors resulted in the identification of at least three protease classes in the crude extract of *C. finmarchicus*. Those classes were serine, metallo and aspartic proteases. The serine and metallo proteases were found to be dominant under neutral to alkaline conditions and the aspartic proteases were dominant under acidic conditions. The cysteine proteases, if at all present, did not seem to be active or present in any substantial amount. The data presented points towards controlling and preserving the protein content in fresh *C. finmarchicus*.

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Keywords: *Calanus finmarchicus*; Proteolytic activity; Protease classes; Inhibitors; Feed; Cod juveniles

1. Introduction

Calanus finmarchicus is a zooplankton species present in enormous amounts in the North Atlantic Sea and is consumed by fish and constitutes a low trophic level in the ocean (Melle and Olsen, 2002). The biochemical composition of some development stadia of this species may be beneficial for fish feed formulations because of its high content of protein and omega-3 fatty acids (Evjemo et al., 2003). At present, much of the protein in fish feed comes from fish meal and other high trophic level sources (Adelizi et al., 1998; Millamena and Golez, 2001). The use of species from a low trophic level as raw material would be more appropriate since much of the biomass and energy has been lost if higher trophic level sources are used.

A potential problem with the use of *C. finmarchicus* as feed ingredient is the high degree of protein degradation post mortem and a subsequent leaching of highly valuable nutrients (Grabner

et al., 1981). Thus, a conservation method would be needed to effectively stabilize the protein content in the raw material.

The use of *C. finmarchicus* in larval feed formulations is being studied. In this feed some proteolytic activity may be beneficial since fish larvae prefer feed that have some partially, predigested peptides in addition to active protease which may facilitate the protein digestion in vivo (Dabrowski and Glogowski, 1977; Kolkovski, 2001). Thus, the ideal is to control the activity; an inactivation initially in the process with a subsequent activation just before feeding. Such a control demands knowledge of the proteases in *C. finmarchicus*.

Proteases are enzymes that cleave peptide bonds with different specificities. The endoproteases hydrolyze the bond internally in the peptide chain, whereas the exoproteases demand a C- or N-terminal, where they cleave off amino acids in addition to di- and tripeptides (Polgár, 1989). The proteases can also be classified as serine, cysteine, aspartate and metallo proteases. This classification is based on the characteristic of the active site. The serine, cysteine and aspartate residues are present in the active site, respectively, and they help facilitate the reaction, whereas metallo protease uses metal ions as a cofactor (Neurath, 1989). The occurrence and relative activity of the different protease classes

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can be identified by the use of specific inhibitors (Beynon and Bond, 2001).

The strategy in this study was to characterize the proteases classes in the crude extract in addition to the behavior of proteolytic activity with temperature and pH. The study of the protease classes was performed by the addition of specific protease inhibitors to a colorimetric protease assay with hemoglobin as substrate. The scope was to identify the different classes of proteolytic enzymes present in *C. finmarchicus*, their relative activities and to evaluate the possibility of controlling protein degradation post mortem.

2. Materials and methods

2.1. Chemicals

The substrate bovine hemoglobin and inhibitors Pepstatin A (isovaleryl-Val-Val-Sta-Ala-Sta) and AEBSF (4-(2-Aminoethyl)benzenesulfonyl fluoride HCl), the standard protein bovine serum albumin (BSA) and the buffers Tris base (2-amino-2-(hydroxymethyl)-1,3-propanediol), CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) and MOPS (4-morpholinopropanesulfonic acid) in addition to the Folin reagent were procured from Sigma–Aldrich. The cysteine protease inhibitor *N*-ethylmaleimide was obtained from Fluka and anhydrous sodium carbonate, trichloroacetic acid and Na₂EDTA (titriplex) came from Merck. The Coomassie Brilliant Blue G-250 solution for the Bio-Rad Protein Assay was purchased from Bio-Rad Laboratories. All other solvents and chemicals were of laboratory grade.

2.2. Animal catch and non-enzymatic analyses

2.2.1. The raw material

Three batches of *C. finmarchicus* were harvested at three different locations in Norway for comparison of chemical composition. The locations were Bergen (May, 2001), Lofoten (June, 2002) and Trondheim (April, 2002). All studies of proteolytic enzymes were performed on a batch of *C. finmarchicus* harvested outside Hitra, April 2004, frozen onboard and stored at –40 °C for later use.

2.2.2. Ash content in *C. finmarchicus*

Samples of *C. finmarchicus* were weighed and dried overnight at 105 °C. The dried material was weighed and then transferred to a Nabertherm oven and combusted at 550 °C overnight. The ash was weighed and determined as percent of dry matter.

2.2.3. Chitin content in *C. finmarchicus*

The fat was removed by lipid extraction according to Bligh and Dyer (1959). A volume of 500 mL methanol and 500 mL chloroform was added to 125 g *C. finmarchicus*. 250 mL deionized water was added and the mixture was shaken for 10 min and then centrifuged at 8000 g for 15 min at room temperature. The lipid phase was removed and used for determination of lipid content. The solid phase was collected and alkaline salts (such as

CaCO₃) in the outer shell of *C. finmarchicus* were removed by adding 1.5 L 2 M HCl. The mixture was stirred for 24 h in a cold room. The acid was removed and the remaining solid was washed with deionized water. Protein was removed by adding 1.5 L 1 M NaOH to the solid and then incubated at 4 °C overnight. The sample was incubated at 100 °C for 8 h and the remaining NaOH was removed. The procedure was repeated with 400 mL 1 M NaOH. Excess NaOH was removed and the resulting dry material was washed with deionized water and ethanol and dried in a desiccator until stable weight was achieved. The content of chitin was determined as percent of dry matter.

2.2.4. Lipid content in *C. finmarchicus*

The volume of the lipid phase described above was measured and 3 mL samples were transferred to glass tubes. The solvents were evaporated at 70 °C under N₂ atmosphere for 30 min. The tubes were cooled in a desiccator and weighed. The lipid content was determined as percent of dry matter.

2.2.5. Protein content in *C. finmarchicus*

Hundred g of frozen *C. finmarchicus* was left to thaw for 1 h. The sample was then homogenized for 10 min in a Waring commercial blender. Ten grams were frozen and later freeze-dried on a Lyovac GTC over night. The nitrogen content in the freeze-dried sample was measured on a NA1500-N/C/S Analyzer. The total protein content was estimated by multiplying the nitrogen percentage with 4.8%W/%N (Evsjemo, 2001).

A volume of 5 mL deionized water was added to 0.25 g of *C. finmarchicus*. The mixture was shaken for 5 min and filtered through a 5 µm Versapor filter. The fat was removed with 0.5 mL methanol and 0.5 mL chloroform and the mixture was centrifuged at 12,000 g for 5 min. The aqueous phase was removed and the concentration of water-soluble proteins was estimated by using the Bio-Rad Protein Assay (Bradford, 1976).

Intact proteins were precipitated by adding 0.25 mL 10% sulphosalicylic acid to 1 mL defatted crude extract. The solution was left for 45 min at 4 °C and then centrifuged at 5000 rpm for 10 min. The supernatant was removed and diluted 1:20 and frozen at –20 °C. The concentration of free amino acids was estimated with HPLC chromatography.

The amount of non-water-soluble protein was estimated by calculating the difference between total protein and the water-soluble proteins and free amino acids.

2.3. Enzymatic analyses

All enzymatic analyses were performed on *C. finmarchicus* caught at Hitra, 2004.

2.3.1. Preparation of *Calanus* crude extract for enzymatic analyses

Deionized water was added to frozen *C. finmarchicus* (Hitra batch, April 2004) (1:1 v/w) and the mixture was allowed to thaw until a liquid slush was achieved (about 30 min at room temperature). The mixture was shaken by hand twice for about 20 s. The icy *C. finmarchicus*–water mixture was centrifuged at 10,000 g for 60 min and the supernatant was removed. The remaining solids were discarded. The extract was filtrated through

glass wool (fiberglass) and subsequently frozen at $-40\text{ }^{\circ}\text{C}$ in 20 mL aliquots.

2.3.2. Protease assay

Aliquots of crude extract were thawed and diluted 1:10. Protein content was measured by the Bio-Rad Protein Assay (Bradford, 1976) and the general protease activity was measured according to Barrett (1972) and Stoknes et al. (1993) with only minor adjustments. A volume of 0.4 mL diluted crude extract was mixed with 1.2 mL 25 mM buffer (see below) and 0.4 mL 1% hemoglobin (Sigma). Zero time samples were made for each sample. The tubes were incubated at $37\text{ }^{\circ}\text{C}$ and the reaction was stopped with 5% trichloroacetic acid (TCA). The tubes were left to cool at room temperature for 30 min. The increase in the concentration of TCA-soluble peptides was measured by the Lowry method (Lowry et al., 1951), and the proteolytic activity was defined as the increase in μg TCA-soluble peptides per min and mg protein. This method was used throughout the study except for the inhibitor study where additional adjustments were made (see below). The incubation time was initiated by the addition of substrate (hemoglobin) and terminated by the addition of TCA.

2.3.3. General proteolytic activity as a function of pH

The proteolytic activity in *C. finmarchicus* crude extract was measured at pH 3, 5, 7, 9 and 11. The buffers used were 25 mM formate, 25 mM piperazine, 25 mM MOPS, 25 mM Tris HCl and 25 mM CAPS, respectively. The incubation time was 3 h.

2.3.4. General proteolytic activity as a function of temperature

The proteolytic activity in *C. finmarchicus* crude extract was measured at 5, 10, 25, 37, 45, 50, 60 and $70\text{ }^{\circ}\text{C}$. The buffer was 25 mM MOPS pH 7. The incubation time was 3 h.

2.3.5. Proteolytic activity as function of temperature in the presence of protease inhibitors

Solid Na_2EDTA was dissolved in crude extract to a concentration of 10 mM. The extract was kept in refrigerator for 30 min before incubation. A volume of 0.4 mL crude extract with 10 mM EDTA was incubated with 1.6 mL 25 mM MOPS pH 7, 10 mM EDTA and 0.2% hemoglobin for 60 min. The assay mixture was incubated at 4, 10, 20, 30, 40, 50, 60 and $70\text{ }^{\circ}\text{C}$ in triplicates. A zero time sample was made by adding 2 mL 5% TCA at the start for each replicate. The reaction was stopped with 2 mL 5% TCA after 60 min. The increase in TCA-soluble peptides was measured as described above.

The same procedure was performed with serine protease inhibitor AEBSF. A concentration of 0.1 mM AEBSF was used in the extract mixture.

2.3.6. Identification of protease classes by specific protease inhibition

The extracts were preincubated for 45 min at $37\text{ }^{\circ}\text{C}$ in the presence of either 0.9 mM 4-(2-Aminoethyl)benzenesulfonyl fluoride HCl (AEBSF), 1 μM Pepstatin A, 10 mM EDTA or 0.8 mM *N*-ethylmaleimide. The remaining proteolytic activity was measured by the hemoglobin colorimetric protease assay described above. Inhibitor was added in excess to ensure full inhibition.

3. Results and discussion

Three different batches of *C. finmarchicus* from 2001 to 2002 were used in this study to give an overview of the major chemical components in some typical batches. A fourth batch caught at Hitra, Norway (2004) was used as raw material for the enzymatic analyses of crude extract and the result was compared with some results from the studies of Antarctic krill. The present paper focus on a different set of enzymes than the report by Knotz et al. (2006) who measured enzyme activities in individual small copepods.

3.1. Animal capture and non-enzymatic analyses

3.1.1. Chemical composition of *C. finmarchicus*

Fig. 1 shows the differences in chemical composition between batches of *C. finmarchicus* caught at three different Norwegian locations. The variation in lipid content can be a result of differences in copepod stadia (Kattner, 1989), i.e. the batches from Bergen and Trondheim might have had larger populations of stadium IV and V with respect to the Lofoten batch and hence a higher content of wax esters. The ash content in the Lofoten batch is particularly high and a large proportion of it may be seawater ions. Differences in harvest methods and handling, i.e., draining time could explain this. However, the relative amounts in chitin and protein are more constant in this study. The sum of the components in Fig. 1 is not 100%. This is probably due to binding of water in the samples, since the high ionic content of *C. finmarchicus* samples generally led to high water-binding capacity. In addition, the lipid content may have been underestimated because of insufficient extraction of polar lipids in the Bligh and Dyer method.

3.2. Proteins in *C. finmarchicus*

It was estimated that about 16% of total protein is water-soluble protein (Bio-Rad) and 24% exists as free amino acids (HPLC). Thus the amount of non-water soluble corresponds to

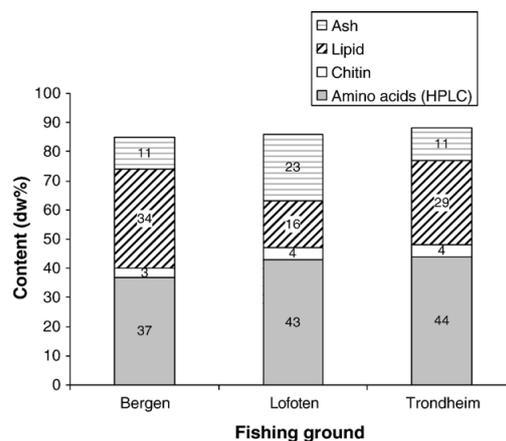


Fig. 1. Chemical composition of three batches of *C. finmarchicus*.

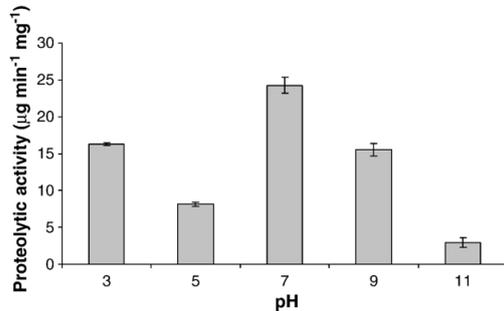


Fig. 2. Specific proteolytic activity in *C. finmarchicus* crude extract as a function of pH. Assay mixtures were incubated at 36 °C for 3 h.

60%. The majority of the amino acids are incorporated into non-water-soluble protein. The water-soluble proteins are quickly degraded and lots of protein would be subsequently lost through leaching if suspended in water as observed by Grabner et al. (1981). In addition, some of the non-water-soluble part could be lost if the raw material is alkali treated, because the proteins become generally more soluble at high pH (Schmittschmitt and Scholtz, 2003).

3.3. Enzymatic analyses

3.3.1. Proteases in *C. finmarchicus* crude extract

The study of proteolytic activity in *C. finmarchicus* was initially performed by monitoring the protein content and amino acid content in homogenized *C. finmarchicus*. Results not included here showed increase in free amino acids and decrease in water-soluble protein as a function of time. However, the results contained a lot of noise making data analysis difficult, especially under alkaline conditions. In addition, the experiments were time consuming and impractical with regard to preparation and sampling. Experiments on krill by Osnes and Mohr (1985a) showed a great deal of similarity between proteolytic activity in homogenate (crushed in blender) and crude extracts. The pattern of amino acid accumulation post mortem in krill varies indifferently between homogenate and crude extract. It can thus be assumed that also

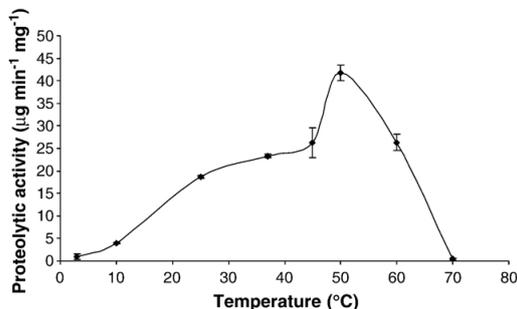


Fig. 3. Specific proteolytic activity in crude extract as function of temperature. Hemoglobin was incubated with crude extract for 3 h at pH 7. Assay mixture (2 mL) contained 0.2% hemoglobin (v/w). The buffer used was 25 mM Mops pH 7.

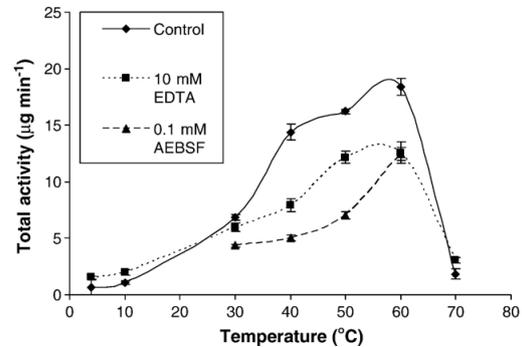


Fig. 4. Proteolytic activity as a function of temperature and inhibitor at pH 7, 25 mM Mops. 1 hour incubation.

crude extracts of *C. finmarchicus* would be suitable for the study of such catabolic processes. Hence, it was decided to use a simpler and more practical model system by studying the proteolytic enzymes in the crude extract of *C. finmarchicus* with the assumption that the proteases present in the crude extract would reflect the proteolytic enzymes in the raw material.

3.3.2. Proteolytic activity as a function of pH

Fig. 2 shows proteolytic activity in crude extract of *C. finmarchicus* as a function of pH from 3 to 11. The error bars are standard errors of the mean (SEM). The columns in Fig. 2 clearly show a proteolytic activity at pH 3, 5, 7 and 9. The low activity detected at pH 11 may be caused by chemical degradation in addition to enzymatic cleavage. However, repeated assays at pH 11 gave variable results as can be seen from the protease class study (shown below). Hence, a pH of 12 may be needed to fully deactivate the trypsins to give a total protease inhibition.

The activity at pH 3 may suggest pepsin-like activity and the activities at pH 7 and 9 may be caused by serine proteases such as chymotrypsin and trypsin-like enzymes in addition to metalloproteases. Possible metalloproteases might be carboxypeptidase A and B which have been detected in krill (Osnes and Mohr, 1986).

3.3.3. Proteolytic activity as a function of temperature

Studies of proteolytic processes in Antarctic krill (Osnes and Mohr, 1985a) show activities at temperatures ranging from 0 to 70 °C. A similar study has been performed with *C. finmarchicus*. The motivation for this experiment was to evaluate the possibility to terminate proteolytic activity by mild thermal treatment.

The activity as a function of temperature at pH 7 is shown in Fig. 3. This pH was chosen because it is the normal pH of *Calanus* after harvesting. A low proteolytic activity was found at 4 and 70 °C. The overall proteolytic optimum temperature was at 50 °C. Osnes and Mohr (1985a) reported temperature optima in krill crude extract to be 50 and 60 °C with incubation times of 10 and 20 min, respectively. The curve in Fig. 4 shows a plateau in the 25 to 45 °C region. This differs from the linear curve reported by Osnes and Mohr (1985a). The incubation time in our study is 180 min. This relative long incubation time was used to improve the sensitivity of the assay. The difference in curvature could hence

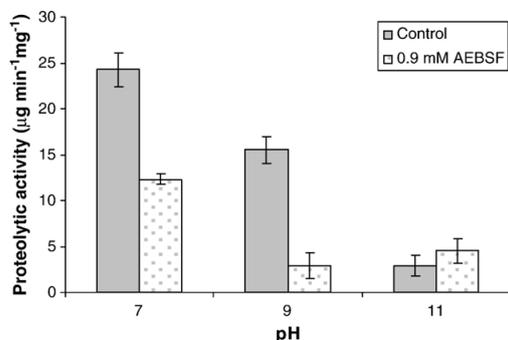


Fig. 5. Effect of serine protease inhibitor on the proteolytic activity in *Calanus* crude extract. The assay mixture was incubated at 37 °C for 3 h. AEBSF 0.9 mM. Assay mixture (2 mL) contained 0.2% hemoglobin (w/v).

be explained by a lack in temperature stability. The proteases with optima between 25 and 45 °C might be less stable and thus show a lower activity after 3 h. A similar study of temperature dependence on crude proteases in krill was done by Yoshitomi (2005). In this study the incubation time was 60 min which gave same curvature as achieved by Osnes and Mohr (1985a). The standard deviations at 45, 50 and 60 °C are significantly larger than at the other temperatures in this 3 hour period. This may reflect greater protease instability in this temperature region. The data show, in spite of these effects, that the optimum temperatures for proteolytic activity in crude extract is significantly higher than the normal working temperature for these enzymes, which would be around 0–10 °C in the north Atlantic sea. However, the fact that Krill show a similar optimum range suggests that this behavior is not limited to *C. finmarchicus*.

3.3.4. Proteolytic activity as a function of temperature and inhibitor

The proteolytic activity at pH 7 is most probably due to serine- and metal protease activity when considering the inhibitor experiments (see discussion below). In addition, the temperature series may suggest two temperature optima. To further enlighten this, two new temperature series were made in the present of either a serine protease inhibitor (AEBSF) or a metal protease inhibitor (EDTA).

Fig. 4 shows that metallo- and serine proteases are both active in 30 to 60 °C region. The decreased incubation time led to a 10 degree higher optimum temperature suggesting some degree of thermal instability. Fig. 4 suggests two temperature optima. The use of inhibitors shows that the optima are the sum of both serine and metalloproteases. However, the sum of the activities at 60 °C does not equal the total activity, which may propose that the inhibitor helps to stabilize the uninhibited protease class by preventing its degradation.

3.3.5. Protease classes in *C. finmarchicus*

The hydrolytic action of proteases in a homogenate of *C. finmarchicus* can be controlled by the addition of protease inhibitors. In addition, specific protease inhibitors directed toward each class of protease are readily available. This gives the

possibility to identify the different protease classes in the crude extract. The concentrations of inhibitors used were based on the recommendations by Sigma–Aldrich. The highest recommended concentrations were used to ensure full inhibition.

3.3.6. Serine proteases

A concentration of 0.9 mM AEBSF in the incubation mixture gives a significant reduction in proteolytic activity at pH 7 and 9 as shown in Fig. 5 and Table 1. The 49% reduction at pH 7 could suggest that about half of the proteolytic activity at 37 °C and pH 7 is caused by the presence of serine proteases. Likewise, the 81% reduction at pH 9 could be caused by the inhibition of serine proteases. However, this suggests that most of the proteolytic activity at 7 and 9 are caused by serine and metallo proteases (see below).

3.3.7. Metalloproteases

The effect of EDTA on proteolytic activity in *Calanus* crude extract is shown in Table 1. A concentration of 10 mM Na₂EDTA in the incubation mixture reduced the proteolytic activity significantly at pH 7 and 9. The highest inhibiting effect was observed at pH 7. The activity was inhibited by 63%, while 41% inhibition was obtained at pH 9. The inhibition of metallo proteases at pH 11 was apparently 94%. As mentioned before, however, the activity detected at this pH varied a lot and this made it difficult to identify the protease classes at this pH.

Metalloproteases like carboxypeptidase A and B have been detected in krill by Osnes and Mohr (1986), where these enzymes were active in the pH range of 6–8. The carboxypeptidases have Zn²⁺ as cofactor and their activity are effectively inhibited by addition of EDTA (Christianson and Lipscomb, 1989).

3.3.8. Cysteine proteases

The effect of cysteine protease inhibitor in *Calanus* crude extract is shown in Table 1. The addition of *N*-Ethylmaleimide to the assay mixture did not significantly reduce the proteolytic activity at pH 7. However, there seems to be an inhibition of

Table 1
General proteolytic activity and inhibition of different protease classes in *C. finmarchicus* at pH 3, 5, 7, 9 and 11

Inhibitor	pH	General activity (µg mg ⁻¹ min ⁻¹)	Activity with inhibitor (µg mg ⁻¹ min ⁻¹)	Fractional inhibition
AEBSF, serine protease	7	24.3±2.1	12.3±0.6	0.49
	9	15.5±1.7	2.9±1.6	0.81
	11	2.9±1.4	4.5±1.5	0*
EDTA, metallo protease	7	25.4±0.3	9.5±0.86	0.63
	9	11.3±4.3	6.6±2.8	0.41
	11	9.6±0.5	0.6±1.8	0.94
Pepstatin A, aspartic protease	7	20.5±2.4	23.7±1.2	0*
	5	8.1±0.6	1.9±0.5	0.77
	3	16.3±0.4	2.5±0.1	0.84
NEM, cysteine protease	7	23.1±7.6	22.6±1.6	0.02
	5	3.4±1.5	1.7±5.3	0.49
	3	13.5±1.5	9.9±1.5	0.27

The results are listed as activity±SEM and the effect is given as fractional inhibition which is the difference between general activity and activity with inhibitor divided by the general activity.

*Observed values were negative.

activity at pH 3. However, the apparent inhibition at pH 5 may not be significant as indicated by the high standard error. The experiment showed 27% inhibition at pH 3, which suggest that the cysteine proteases would only be important at low pH. In any case, the cysteine proteases do not seem to present any substantial amount.

3.3.9. Aspartic proteases

Table 1 gives the result from the inhibition study with pepstatin A. The activity is significantly reduced at both pH 3 and 5 by approximately 84 and 77%, respectively, while pepstatin A seems to activate the proteases at pH 7. However, the standard error in the control shows that the activity in *Calanus* crude extract at pH 7 with or without Pepstatin A is not significantly different. This suggests that the aspartic protease activity operates below pH 7, which is to be expected, and that this activity would only contribute significantly if the raw material is processed at acidic condition. The activity at pH 3 may be caused by pepsin-like enzymes, which have a pH optimum around pH 2 and are normally activated below pH 5 (Stryer, 1995).

3.3.10. Overview of protease class experiments

The inhibitor experiments on protease classes were performed to get a rough picture of the range of proteases in *C. finmarchicus*. The experiments suggest that at least three protease classes are present in substantial amounts. This is not surprising considering the wide range of proteases revealed in krill by Osnes and Mohr (1985b, 1986).

The results shown in Fig. 6 suggest that the aspartic proteases are dominant under acidic conditions whereas both the metallo and serine proteases are dominant under neutral to alkaline conditions. The cysteine proteases seem only to be present in lower amounts at low pH. Thus, mainly aspartic proteases have to be controlled at pH below physiologic conditions if protein degradation is to be minimized. Both metallo and serine proteases are active at neutral and alkaline conditions. They can be considered equally important groups of proteases.

The summation of activities in Fig. 6 resembles the activities shown in Fig. 2. The summed activities are generally higher than the total activity measured at the same pH. This may suggest increased protease stability as mentioned earlier (Fig. 3). The

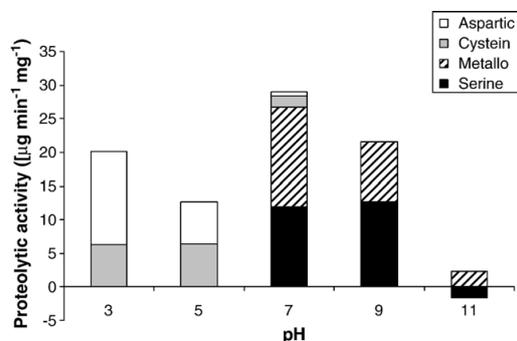


Fig. 6. Relative amounts of protease classes as a function of pH.

inhibition of one protease class could perhaps stabilize the other uninhibited class against degradation, and thereby exhibiting a higher total activity.

The column at pH 11 shown in Fig. 6 is the result of an unsuccessful determination of proteolytic activity at this pH. As mentioned earlier, there may be other chemical processes that occur in a high alkaline milieu. In addition, the hemoglobin colorimetric protease assay may not be applicable at this condition. However, disregarding the above mentioned uncertainties at this high pH, it would be reasonable to believe that the metallo proteases would be the protease class responsible for any proteolytic activity at this pH.

4. Conclusion

At least three classes of proteases appear to be present in crude extract of *C. finmarchicus*. The aspartic proteases are dominant at acidic conditions, whereas the metallo and serine proteases are both present in substantial amounts at alkaline conditions. The aspartic proteases are probably pepsin-like enzymes. The serine proteases are most likely trypsin-like enzymes and the metallo proteases are probably carboxypeptidase-like enzymes.

The proteolytic activity is relatively low at 4 to 10 °C. The optimum was found to be 50 °C with 3 h incubation time. An incubation time of 1 h shifted the optimum up to 60 °C. This may reflect protease instability with longer incubation times. The activity is low at 70 °C.

The results in this study are important to understand the degradation of protein in *C. finmarchicus*. Knowledge of the classes of protease and temperature dependencies gives a good starting point for establishing a strategy for protein conservation control. Production of *Calanus*-based feed at moderate alkaline condition would demand control over the serine- and metallo proteases, whereas mainly the aspartic proteases would be necessary to control under acidic condition, since the cysteine proteases, if at all present, most probably play a minor role. A careful use of natural protease inhibitors in the feed may help prevent protein degradation, i.e. introducing soybean (*Glycine max*) inhibitors could deactivate the serine protease class (Fennema, 1996). However, care must be taken because the presence of inhibitors may inhibit the proteases in the fish juvenile digestive channel and thereby significantly reduce the protein digestibility of the feed. Alternatively, as the temperature study shows, a heat treatment at 70 °C or above would be sufficient to inactivate most of the proteases. Hence, control of proteolytic activity by the use of heat may be more feasible for large scale bulk production of *C. finmarchicus* protein meal.

On the basis of the results in this paper it would be interesting to investigate the proteases in more detail and compare them to krill proteases from both a biochemical and phylogenetic point of view.

Acknowledgments

This work was supported by The Norwegian Research Council through Codtech, Larval feed components (project no. 81189300). The authors would like to thank assistant professor T. Rustad, Department of Biotechnology, NTNU, for suggesting

appropriate protease inhibitors for this study. Thanks also to the members of the *Calanus* project for supplying the raw material.

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Paper II



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Food and Bioproducts Processing



Encapsulation of a proteolytically active novel bioproduct; controlling the release of proteinous components

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ARTICLE INFO

Article history:
Received 10 January 2008
Accepted 12 March 2008

Keywords:

Marine bioproduct
Alginate
Protein release
Protein–alginate interactions
Protein conservation methods

ABSTRACT

Low trophic level organisms like zooplankton not only represent a valuable and unutilized source for marine proteins and lipids, but are also challenging with respect to high post-mortem protease activity. As an example, Ca–alginate encapsulated homogenized fresh zooplankton (*Calanus finmarchicus*) exhibited a high release of protein components when immersed in water due to diffusion of proteolytically degraded proteins. Initial diffusion rates at pH 7 increased with temperature up to 60 °C. Above 50 °C, the release was reduced because of protease instability after 12 h. The release of protein also increased with increasing ionic strength, most likely due to decreased electrostatic interaction between the alginate matrix and protein. As function of pH, the release of both amino groups as well as larger protein entities was apparently highest under alkaline conditions. Encapsulated heat-treated or alkali treated zooplankton had a high degree of release, the first 2 h reflecting the presence of osmoregulating amino acids as well as pre-digested proteins. After 2 h, no further release of protein was observed, which can be attributed to protease inactivation caused by these treatments. The present data show that all studied parameters (temperature, pH and ionic strength) have a profound impact on protein loss from the encapsulated model feed particles. Therefore, possible conservation methods to control the observed protein loss from marine raw materials in, e.g. marine feed formulations are suggested.

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1. Introduction

Calanus finmarchicus is a low trophic level zooplankton species, which easily can be harvested and is present in massive amounts in the northern oceans (Melle and Olsen, 2002). This species has a high-protein content and a beneficial fatty acid profile (Solgaard et al., 2007), and may therefore be a new, useful resource in human and animal nutrition. For example, Ca–alginate-gelled particles containing *C. finmarchicus* may be used as, e.g. feed for marine juveniles as a replacement for *Artemia* and Rotifers. The zooplankton may also be an appropriate fish meal substitute on a general basis. At present, however, most of the protein in fish feed comes from fish and other high trophic level sources (Adelizi et al., 1998; Millamena and Golez, 2001), which is becoming an increasingly limited resource for this purpose. More generally, protein and lipids

derived from *C. finmarchicus* may also serve as useful additives in food and nutraceutical products to improve their nutritional value (e.g. functional foods). Thus, *C. finmarchicus*, as a low trophic species, could be considered to be an attractive resource in a world with a growing demand for marine proteins and lipids; both for human consumption as well as for feed to farmed animals.

However, a high catabolic activity was observed in crude extracts of *C. finmarchicus* post mortem (Solgaard et al., 2007) resulting in a high degree of protein loss as function of time. Most of the water-soluble protein is rapidly digested and turned into small peptides and amino acids. Some enzyme activity may, however, be beneficial under certain circumstances as it, e.g. facilitates digestion in juvenile marine larvae (Kolkovski, 2001; Dabrowski and Glogowski, 1977). Thus, the primary goal for the exploitation of these bioproducts is to be able to control the overall proteolytic activity; both through a

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doi:10.1016/j.fbp.2008.03.003

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knowledge of the different proteases present, possible preservation methods as well as available ways of administration of final products, e.g. by encapsulation in a polyelectrolyte matrix exploiting electrostatic interaction and stabilization.

Alginate, extensively used as a matrix for encapsulation, is a polysaccharide composed of $\beta(1 \rightarrow 4)$ -D-mannuronic acid (M) and $\alpha(1 \rightarrow 4)$ -L-guluronic acid (G) and is mainly isolated from brown algae (Draget et al., 2006). This polysaccharide yields temperature stable gels in the presence of some divalent metal ions (e.g. Ca^{2+}). The gel strength is dependent on G content (F_G) and the sequence of these residues (G Block length). Alginate is widely used as a thickening agent in food and in immobilization of enzymes and cells (Draget et al., 2006). Protein-polysaccharide interactions have been extensively studied (Tolstoguzov, 1986; Neiser et al., 1998; Haug, 2003). Proteins and charged polysaccharides may interact through electrostatic interactions depending on factors like ionic strength and pH. As an example, bovine serum albumin (BSA) and alginate interact through electrostatic attractive forces when the pH is below the pI of the protein and above the pK_a of alginate (Neiser et al., 1998). Changes in pH affect molecular charge distribution whereas increasing the ionic strength leads to electrostatic shielding effects as well as a general ion exchange.

This study reports on a complex marine bioproduct, in which the encapsulated material contains a wide variety of biological components. The scope of this study was to investigate the nature of, and the possibility to control, the release of protein components (free amino acids, oligopeptides and larger protein entities) from Ca-alginate encapsulated homogenized *C. finmarchicus* through the manipulation of pH, ionic strength and temperature.

2. Materials and methods

2.1. Chemicals

Lithium hydroxide monohydrate p.a. was purchased from both Merck and Fluka. Potassium hydroxide pellets p.a. and ninhydrin p.a. were procured from Merck and the formic acid (85%) was purchased from Norsk Hydro. Hydrindantin dehydrate p.a. was procured from Fluka. The Na-alginate (Protanal® LF 10/60) was a kind gift from FMC Biopolymer with an average molecular weight of 108 kDa (as determined from light scattering, Vold et al., 2006), and the fraction of guluronic acid residues was F_G was 0.66 (based on ^1H NMR data) with an average G-block length longer than 1 ($\text{NG} > 1$) of 14 (Grasdalen et al., 1979). The calcium carbonate (Eskal 500) was purchased from KSL Staubetechnik gmbh. Coomassie® Brilliant Blue G-250 solutions for the Bio-Rad Protein Assay was procured from Bio-Rad Laboratories and the acetic acid (laboratory grade) was purchased from J.T. Baker. The standard protein BSA, the amino acid standard glycine and the buffers Trizma base (2-amino-2-(hydroxymethyl)-1,3-propanediol), CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) and MOPS (4-morpholinepropanesulfonic acid) were all procured from Sigma. All other solvents and chemicals were of laboratory grade.

2.2. Raw material

The raw material used in this study was *C. finmarchicus* caught in the sea outside Frøya, Norway in May 2006. Parts of the

catch were heat treated at 70–80 °C for 15 min to deactivate proteolytic enzymes, whereas another part of the catch was alkali treated with potassium hydroxide to eliminate proteolytic activity (pH 11). The rest of the batch was untreated, immediately frozen on dry ice and stored at -40 °C for later use.

2.3. Encapsulation of *C. finmarchicus*

Samples of *C. finmarchicus* were thawed for 2 h at room temperature, and homogenized in a Waring® commercial blender for 10 min. To ensure proper stabilization of the alginate as well as keeping CaCO_3 non-dissolved the pH of the homogenized *Calanus* was adjusted to alkaline by KOH shortly before encapsulation. 250 mg solid calcium carbonate per 100 g *Calanus* was slurred in 5 ml deionized water and added to the *Calanus*. A 5% solution of Protanal® alginate LF 10/60 was added to the homogenate to a final concentration of 1% (w/v). The alginate-*Calanus* mixture was stirred for 15 min. As a feed model system, small droplets of the mixture were dripped manually into a 5% HCOOH bath with the aid of a 20 ml syringe (1 mm nozzle) leading to an internally set feed model particle, and the particles were left to harden for approximately 15 min. The average particle size was 3 ± 0.5 mm. The particles were rinsed with 50 mM Mops pH 7 to remove any excess formic acid. Encapsulation was performed in cold room at 4 °C. After preparation, the resulting particles were immediately frozen in 50 mM Mops pH 7 at -40 °C.

2.4. Release of proteinous components

Released proteinous components in each experiment was monitored as a function of time at different conditions in a temperature controlled water bath (Heto SBD50) with mild shaking (50 rpm) to prevent diffusion limitation by film formation. Release values were normalized against encapsulated wet weight material and are reported in this paper as either μmol amino groups per g wet weight or $\text{mg}/\mu\text{g}$ protein per g wet weight.

2.4.1. Release as a function of temperature and time

The alginate encapsulated model particles were thawed and rinsed with 50 mM Mops pH 7. About ten gram particles were transferred to a plastic cup and 50 ml 50 mM Mops was added. The containers were incubated in a water bath at 4, 25, 30, 40, 50, 60 and 70 °C. Samples were taken after 0, 0.5, 1, 2, 4, 8, 12 and 24 h of incubation.

2.4.2. Release at different pH

The alginate particles were thawed and rinsed with incubation buffer (see below). Approximately 7 g of particles was transferred to a plastic cup, 50 ml 50 mM buffer (see below) was added and the particles were incubated at 40 °C. Samples were taken after 0, 0.5, 1, 2, 4, 8, 12 and 24 h. The pHs studied were 3, 5, 7, 9 and 11. The incubation buffers used were 50 mM formate, acetate, Mops, Tris-HCl and Caps, respectively.

2.4.3. Release at different ionic strengths

The feed particles were incubated in 50 mM acetate pH 5 at 40 °C. The different sodium chloride concentrations used were 0, 0.1, 0.3 and 0.5 M. These experiments were otherwise performed as described above.

2.5. Analytical methods

2.5.1. Ninhydrin method for the quantification of released amino groups

The ninhydrin method was performed according to earlier published methods (Moore and Stein, 1948; Prochazkova et al., 1999) with some slight modifications. The ninhydrin assay, used in this study, was performed as follows: 75 ml dimethylsulfoxide (DMSO) was flushed with N₂. Two grams of ninhydrin and 0.3 g hydratindatin were added. The solution was again flushed with nitrogen until all solids were dissolved. Twenty-five millilitres of 4 M lithium-acetate buffer pH 5.2 was added carefully and the resulting dark solution was flushed with nitrogen for 5 min. The reagent was stored in a dark bottle and used within 3 days.

One millilitre of ninhydrin reagent was added to 1 ml sample solution in triplicates. The tubes were covered and incubated for 30 min at 100 °C. The tubes were cooled to 30 °C in a water bath and 5 ml 20% (v/v) ethanol/water was added to each sample. The samples were shaken on a Vortex mixer for 5 s. The absorbance at 570 nm was measured on a Shimadzu spectrophotometer. Glycine was used as a standard.

2.5.2. Protein concentration

The concentration of intact or large protein entities (>3 kDa) was estimated using the Bio-Rad Protein Assay (Bradford, 1976). The absorbance at 595 nm was measured on a PerkinElmer Lambda UV-vis spectrophotometer. Bovine serum albumin was used as standard.

3. Results and discussion

3.1. The Ninhydrin assay

As expected, glycine gave a significantly higher color development than BSA (Fig. 1). The slope (dA/dc) for glycine was approximately 23 times larger than the corresponding slope for BSA, which implies that the ninhydrin method is more sensitive to free amino acids than proteins due to a considerable higher content of free amino acids. Ninhydrin may, however, also react with other molecules containing amino groups, but it may be assumed that free amino acids and oligopeptides were responsible for most of the colorimetric yield in these experiments. As discussed later, some release of NH₃ may have interfered with the method.

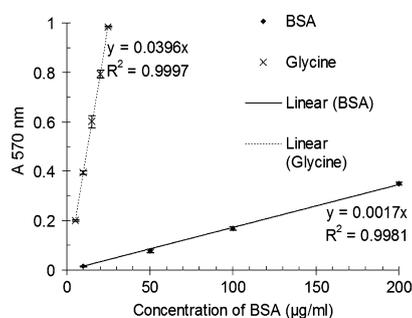


Fig. 1 – Absorbance at 570 nm as function of concentration of glycine and BSA. The error bars represent SDM.

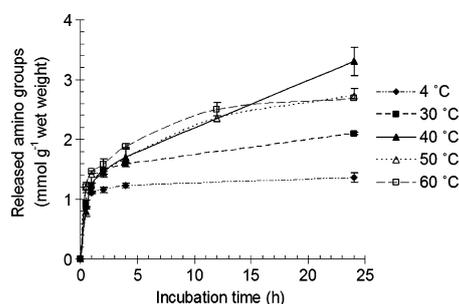


Fig. 2 – Release of amino groups as a function of time at five different temperatures. Buffer was 50 mM Mops, pH 7. The error bars represent SDM.

3.2. Release of amino groups at different temperatures

Fig. 2 shows the increase in the amount of amino groups in the incubation buffer at different temperatures as measured by the ninhydrin method. Ninhydrin solution reacts primarily with primary amino groups on proteins and amino acids but can also react with ammonia and with other amino groups on proteins and other molecules (Friedman, 2004). However, protein hydrolysis will inevitably create more ninhydrin reactive primary amino groups implying that the colorimetric response will increase with increasing protein degradation. It can thus be assumed that the data in Fig. 2 reflects the release of free amino acids and oligopeptides.

At all temperatures, there was initially a high release of amino groups from the encapsulated material within the first two hours of incubation. This initial increase can be attributed to the release of free amino acids, which are present in zooplankton as osmoregulators (Helland et al., 2003), and from pre-digested protein before the catch was frozen. This release of small components is mainly diffusion limited, and such small molecules diffuse very rapidly within an alginate network. It has been found that a molecular weight of more than 300 kDa is needed for globular protein molecules to exhibit diffusion constants lower than that of pure water (Martinsen et al., 1992). However, the particles will also contain active proteases which over time will degrade the water soluble proteins and on a later stage probably also the non-water soluble proteins from the *Calanus* (Solgaard et al., 2007). The latter process is significantly slower and the release after 2 h can thus be regarded as limited by this proteolytic action. The initial protease substrate was most likely the water-soluble proteins, followed by the degradation of the non-soluble proteins when the protease's access to water soluble substrate became limited.

The rate of release was significantly lower after two hours for all temperatures; however, a clear temperature dependency was observed, as expected, since enzymes work faster at higher temperatures. The release rate increased with temperature up to 60 °C, however, a reduced rate was observed at 50 and 60 °C after approximately 12 h of incubation, probably reflecting protease instability (denaturation) at these relative high temperatures (Solgaard et al., 2007). As expected, the lowest rate of release was observed at 4 °C since the proteolytic activity here is known to be low (Solgaard et al., 2007). This result demonstrates the importance of storing thawed *Calanus* as well as fresh feed particles at low temperatures (below 4 °C) to avoid an unacceptable loss of protein.

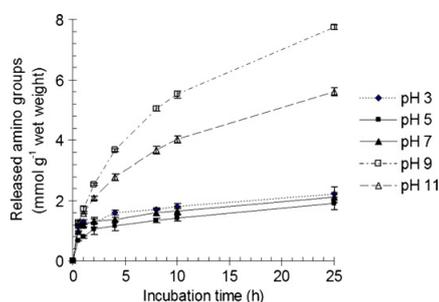


Fig. 3 – Release of amino groups as a function of time and pH. The incubation temperature was 40 °C. The error bars represent SDM.

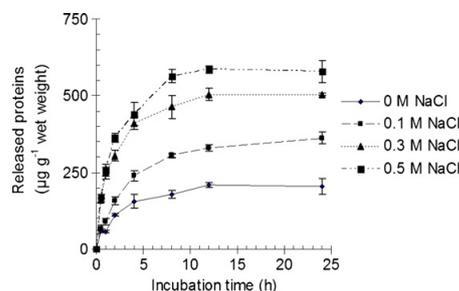


Fig. 4 – Release of proteins (>3 kDa) as function of incubation time and NaCl concentration. Incubation temperature was 40 °C and the incubation medium was 50 mM acetate, pH 5. The error bars represent SDM.

It is likely that both exo- and endo proteases were active in the model feed particles. It is reasonable to anticipate that the exo-proteases were more responsible for the accumulation of free amino acids in the system since they release free amino acids, tri- and dipeptides from the terminal ends of the proteins (Neurath, 1964; Neurath, 1999). However, the endo proteases are indirectly involved since they increase the amount of accessible substrate for the exo-proteases.

3.3. Release of amino groups at different pH

The incubation temperature in these experiments was set to 40 °C to amplify the rate of release.

Fig. 3 shows the release of amino group containing molecules at different pH and shows that there is a high degree of release at pH 9 and 11, apparently suggesting significant exo-activity. The highest release was at pH 9, supporting earlier data that the activity of metallo and serine proteases is considerably higher at pH 9 than at pH 11 (Solgaard et al., 2007), assuming a co-release of proteases into the medium (see also Section 3.5).

At pH 11 however, there was most probably a release of NH₃ from the feed particles due to deamidation of asparagine and glutamine. NH₃ will interfere with ninhydrin assay (Friedman, 2004) and over-estimate the amount of amino acids released. Based on earlier results (Solgaard et al., 2007) there is no reason to anticipate a high protease activity at this pH.

3.4. Release of proteins at different ionic strengths

Feed model particles were incubated for 24 h in 50 mM acetate pH 5 at different salt concentrations. pH 5 was chosen to maximize the electrostatic interactions between proteins and the alginate poly-anion, and again the incubation temperature was again set to 40 °C to amplify the rate of release.

Fig. 4 shows an increasing protein release with increasing ionic strength. At pH 5, the carboxyl group of alginate will carry a negative electrostatic charge, $pK_a \sim 3.5$ (Draget et al., 2006). This opens up for electrostatic attractive interaction to take place between the alginate network and positively charged patches on the water-soluble proteins at low ionic strengths. More specifically, one possibility would be that the ϵ -amino group of the lysine residues makes salt bridges with carboxyl groups. However, an addition of NaCl would weaken such a bond due to the shielding off of short-range attractive forces (Neiser et al., 1998) as well as reducing the entropic driving

force of the release of condensed counter-ions. The net result will be an increased release of proteins from the particles at this high ionic strength.

3.5. Release of protein as a function of pH

Figs. 3 and 5 show a high degree of release at pH 9 and 11 compared to pH 7. This may partly have been due to an increased solubility of protein at higher pH (Schmittschmitt and Scholtz, 2003). The data in Fig. 3 suggest that the highest degree of release is at pH 9 whereas the data in Fig. 5 apparently show a higher release at pH 11. However, it is likely that there was also a co-release of proteolytic enzymes into the surrounding medium, which subsequently would degrade released proteins into small peptides and amino acids efficiently at pH 9. Thus, Fig. 5 reflects both release of proteins from alginate encapsulated *Calanus* at pH 9 and 11 as well as proteolytic activity in the surrounding medium at pH 9 leading to low molecular weight proteinous material not detectable with the Bio-Rad method (Bradford, 1976). Indeed, the apparent reduction of released proteins with time, at pH 9 (Fig. 5), supports the view that such proteolytic degradation took place.

The increased release at pH 9 and 11 (Fig. 5) was most likely due to electrostatic repulsion between the negatively charged alginate network and proteins carrying a net negative charge at high pH. This may also explain why there was a reduced release at neutral and acidic conditions.

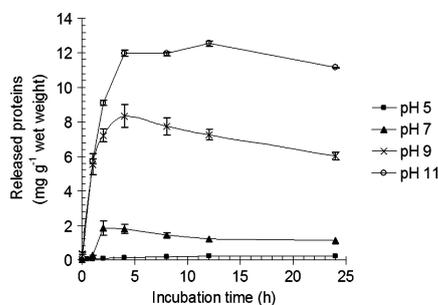


Fig. 5 – Release of proteins (>3 kDa) as a function of time and pH. Incubation temperature was 40 °C. The error bars represent SDM.

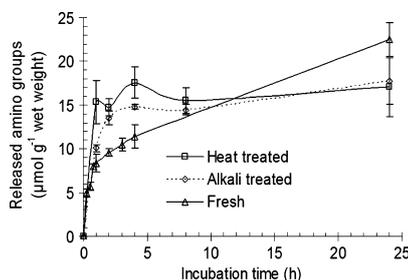


Fig. 6 – Release of amino groups from feed particles made of heat treated, fresh and alkali treated *C. finmarchicus*. The incubation medium was 50 mM Mops, pH 7. Incubation temperature was 40 °C. The error bars represent SDM.

3.6. Release from particles made from heat and alkali treated complex bioproduct

In order to examine the possibility of controlling the release of proteinous material from model particles two different preservation methods were compared with encapsulated fresh material (Figs. 6 and 7).

Fig. 6 shows the release of amino groups from models feed particles made from heat-treated, alkali treated and fresh *C. finmarchicus*. The level of free amino groups in the heat-treated sample is around 18–19 µmol per g wet weight after only one hour of incubation, suggesting that there is a high initial content of free amino acids in the heat-treated *C. finmarchicus*. Since no further release is observed, it is reasonable to anticipate that the proteases have been inactivated. The high release of amino groups from the heat-treated *C. finmarchicus* is partly due to the presence of osmoregulating amino acids, but also caused by an accelerated protease activity early in the heating process. However, the data also suggest that most of the proteolytic activity has been lost after treatment at 80 °C for 15 min. A rapid heating process is therefore recommended to avoid loss of protein components during this preservation method.

The release of amino groups from feed particles of alkali treated *C. finmarchicus* is also high the first two hours, again reflecting the inherent content of free amino acids, but also suggesting that there may have been some degree of protein hydrolysis in the alkali treated *C. finmarchicus*. In addition, the high pH (11) may have altered some non-water soluble

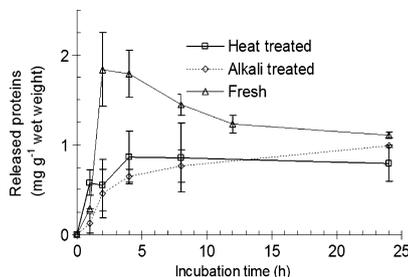


Fig. 7 – Release of proteins (>3 kDa) from feed particles made of heat treated, fresh and alkali treated *C. finmarchicus*. The incubation medium was 50 mM Mops, pH 7. Incubation temperature was 40 °C. The error bars represent SDM.

proteins into water-soluble molecules, and there may have been some release of NH₃, which would react with ninhydrin method.

Fig. 7 shows the release of proteins from feed particles made from heat-treated, alkali treated and fresh (untreated) *C. finmarchicus*, showing a significant difference in the release of proteins between treated and untreated samples. Particles of fresh biomaterial release more than twice as much protein during the first 2 h followed by a clear decrease in medium protein content after 4 h, again suggesting proteolytic activity in the medium. The heated and alkali treated particles show no significant decrease in medium protein, confirming that most of the protease activity has been lost. In essence, Figs. 6 and 7 show that fresh model feed particles leach more large protein entities and show an increasing concentration of free amino acids and oligopeptides over time due to intact proteolytic enzymes than do alkali and heat-treated biological material.

4. Conclusions

A high degree of amino group release from alginate encapsulated *Calanus* particles was observed, and increased with temperature up to 60 °C, caused by an increased proteolytic activity. After 12 h, the release was inhibited at temperatures above 50 °C due to an inactivation of the proteolytic enzymes. Liberation of protein increased with increasing ionic strength, mainly caused by a weakening of the electrostatic interaction between the alginate poly-anion and the proteins. As function of pH, the release of amino groups was highest at pH 9, whereas release of proteins was apparently highest at pH 11. However, proteolytic activity in the medium at pH 9 was probably degrading the proteins leading to an increase of free amino acids. Model feed particles manufactured from heat or alkali treated *C. finmarchicus* had, as expected, a high initial release of proteinous material. The amount of release from such particles leveled off after two hours, indicating protease deactivation, suggesting these two possible preservation methods to be adequate for protein maintenance in the present encapsulated system.

This study has shown that encapsulation of fresh zooplankton gives particles that release high amounts of proteinous components. The rate and quantity of protein loss is dependent on factors like pH, temperature and ionic strength. The simplest way to reduce protein loss is by heat or alkali treatment of the raw material to inactivate the proteases. The present data suggest that the interactions, which have been shown to govern simpler systems (George and Abraham, 2006), also seem to govern the major processes in the complex and dynamic system presented here.

In the case of feed particles, it may therefore be concluded that the inherent presence of free amino acids may act as attractants, whereas a protease inactivation ensures sufficient nutritional value. Inactivation by heat treatment may be preferred prior to alkaline treatment since the latter can cause a higher loss of large protein entities due to increased solubility.

Acknowledgements

We thank Prof. Olav Vadstein, Prof. Kjell M. Vårum, and Prof. Turid Rustad for valuable discussions. This work was supported by the Norwegian Research Council through the

Strategic University Program "CODTECH – A process oriented approach to intensive production of marine juveniles with main emphasis on cod" (project no.153422). Members of the Calanus project (CALANUS – Exploitation of zooplankton as bio-resource for fish feed and industrial raw material – A complete value chain evaluation, 331 project no. 143184), Nils Tokle and Nils Melhuus are thanked for giving us the opportunity to catch Calanus in the spring 2006 at Hitra/Frøya, Norway.

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Paper III



Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Proteolytic degradation of cold-water fish gelatin solutions and gels

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ARTICLE INFO

Article history:

Received 25 March 2008

Received in revised form 6 May 2008

Accepted 8 May 2008

Available online 16 May 2008

Keywords:

Fish gelatin

Proteolytic degradation

Rheology

ABSTRACT

The stability of cold-water fish gelatin (FG), both in solution and in the gel phase, has been studied as function of both temperature and exposure towards novel proteases of marine origin. A 1% (w/v) FG solution was readily degraded by such proteases above 20 °C, which was expected since FG at this temperature is a random coil molecule lacking the protective triple helical structure found in collagen. The dynamic storage modulus for a 10% (w/v) FG gel increased monotonically at 4 °C. Ramping the temperature to 6, 8 or 10 °C led to a drastic reduction in G' , but an apparent partial recovery of the network (increasing G') was observed with time at all temperatures. In the presence of proteases, a lower storage modulus was observed. At constant 4 °C, an apparent maximum value was reached after curing for 2 h followed by a decrease in G' indicating protease activity. Ramping of temperature in the presence of proteases led to an even more drastic reduction in G' and no recovery of structure was observed with time. In this case, the overall rheological behaviour is a complex function of both thermal influence as well as proteolytic activity. In an endeavour to quantify the effect of the presence of proteolytic enzymes on the gelatin network, rheological investigation were undertaken where the dynamic storage moduli were recorded on different 10% (w/v) FG samples that had been acid hydrolysed to yield different average molecular weights. A significant reduction in storage modulus for average molecular weights below 50 kDa was found. This critical molecular weight most probably reflects the on-set of a regime where shorter chain lengths prevent percolation due to an increase in the loose end and sol fraction as well as a reduction in the average length of the pyrrolidine-rich regions reducing the number of possible junction zones.

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1. Introduction

Gelatin is a biomacromolecule derived from the fibrous protein collagen, and may be extracted from skin and bone tissue after acid or alkali pre-treatment [1–4]. Traditionally, sources of gelatin have been mammalian species such as cattle and pigs. However, marine species such as Cod (*Gadus Morhua*), Pollock (*Polachius virens*) and Haddock (*Melanogrammus aeglefinus*) have been used for fish gelatin (FG) manufacturing for the last three decades, and the chemical and physical properties of these gelatins have been increasingly studied during the last decades [5–7]. In 2006, the worldwide production of gelatin was reported to be approximately 315,000 tonnes, of which FGs constituted less than 2% (<http://www.gelatine.org>).

The main difference between cold-water FG and mammalian gelatin has been identified to be the various contents of the imino acids proline and hydroxyproline [4,5,8,9]. Because of this, cold-water FG has sub-optimal physical properties compared to mammalian gelatin in applications [1]. The gelling and melting

temperatures of gelatins have been found to correlate with the proportion of proline and hydroxyproline in the original collagen. Cold-water FG has typically been found to gel at 4–5 °C and melt at 12–13 °C [1], whereas bovine gelatin sets and melts at approximately 22–24 and 31–33 °C, respectively [1,10,11].

When extracted collagen is heated above the denaturation temperature, the triple helices unwind and the collagen turns into random coil gelatin. Upon cooling a gelatin solution, some of the triple helical collagen structure is regained, but 100% regeneration of collagen structure has never been reported [12–14]. The pyrrolidine-rich regions in the gelatin α -chains act upon cooling as nucleation sites for formation of triple helical junction zones leading to a gel network [15,16]. The length of these junction zones is proposed to be at least 20–30 amino acids, and they are interconnected by flexible peptide chains, which are known as elastic segments [15]. The junction zones are believed to be stabilised by hydrogen bonds similar to those in native collagen, and the junction zones are mainly composed of individual triple helices [11]. However, some of the junctions may also consist of several, aggregated triple helices [8,17].

The use of FG as an encapsulating agent may be of interest in, e.g. feed formulations for newly hatched marine fish larvae since the low melting temperature of cold-water FGs may facilitate the

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digestion and simultaneously act as a protein and energy source. Mechanical stability is, however, a crucial parameter in such an application. This is particularly important when using protease-containing zooplankton such as *Calanus finmarchicus*, as a basic feed constituent. Proteases are known to easily attack and degrade gelatin in solution where the gelatin molecules are present as random coils. The proteolytic degradation of gelatin is however decelerated or inhibited in gelatin gels where parts of the molecule exist in a triple helical structure [18].

C. finmarchicus is yet a non-utilised resource for feed manufacturing, but is considered to be well suited due to the vast amounts present and its lipid and protein content [19,20]. In addition, the feed has to be stable at temperatures $\leq 12^\circ\text{C}$, since 12°C is the typical water temperature used in the farming of, e.g. cod larvae [21].

The scope of this paper was to study the mechanical stability of FG solutions and gels affected by changes in temperature and in the presence of proteases from *C. finmarchicus* applying rheological methods. The time-resolved mechanical properties of the gelatin gel as function of proteolytic degradation was tentatively correlated to the properties of gels made from hydrolysed gelatin fractions with known molecular weights. It should be pointed out, however, that a study of this kind points far beyond the use of cold-water FG as a matrix in feed formulations; the results obtained may be of value for any use of any type of gelatin where proteases are present.

2. Materials and methods

2.1. Chemicals and sample preparation

2.1.1. Chemicals

Trizma base (2-amino-2-(hydroxymethyl)-1,3-propanediol) and standard protein bovine serum albumin (BSA—Fraction V, $\geq 96\%$, A9647) were purchased from Sigma-Aldrich and the Coomassie® Brilliant Blue G-250 solution for the Bio-Rad Protein Assay was procured from Bio-Rad Laboratories. The hydrochloric acid was purchased from Merck.

2.1.2. Fish gelatin

The FG was kindly provided by Norland Products Inc., USA. Norland (Lot #6120 and #4177) and produced from the skins of cold-water fish species such as Cod, Haddock and Pollack. The molecular weight was in the range 120–130 kDa and the intrinsic viscosity was approximately 40–42 ml/g as determined from Size Exclusion Multi-Angle Laser Light Scattering (SEC MALLS) and capillary viscometry, respectively.

2.1.3. FG degradation by acid hydrolysis

FG (15% (w/v) in 0.1 M HCl) was hydrolysed at 60°C for up to a maximum of 13 h. Samples were collected at different time intervals, cooled in ice water to room temperature (22°C), and neutralised with 1 M NaOH. After depolymerisation and neutralisation, the samples were dried by lyophilisation and stored at -40°C prior to application. FG samples with weight-average molecular weights of 28, 35, 40, 42, 51, 74 and 117 kDa (determined by SEC-MALLS, see Section 2.2.3) were achieved.

2.1.4. Preparation of *Calanus* crude extract

C. finmarchicus was caught and frozen at Frøya, Norway in May 2006. Deionised water was added (1:1, w/w) to half-thawed *C. finmarchicus* (at $\sim 23^\circ\text{C}$, 2 h), and the mixture was centrifuged at 4°C at $16,000 \times g$ for 1 h. The supernatant was filtered, and the protein concentration was measured by the Bio-Rad method [22]

using BSA as a standard. The filtrate was frozen at -40°C for later use, and will in the following be referred to as the *Calanus* crude extract.

2.2. Analytical methods

2.2.1. Capillary viscometry

FG solutions (15 ml, 1% (w/v) in 50 mM Tris-HCl, pH 7.5) were transferred to a Schott-Geräte Ubbelohde (Type 53101/0a). *Calanus* crude extract (35 μl , 3 mg/ml crude protein) was added and the flow-through times were recorded. The specific viscosity (η_{sp}) was determined, and $1/\eta_{\text{sp}}$ was plotted as function of time for 20, 30, 40 and 50°C . Experimental limitations prevented viscosity measurements below 20°C . All calculations were based on FG solutions of relative viscosity $\eta_{\text{rel}} \leq 2$. Tris-HCl buffer (50 mM, pH 7.5) was used as reference solution. The Arrhenius activation energy (E_a) was calculated based on the data obtained for 20, 30 and 40°C , by applying linear regression and using Eq. (1):

$$k' = A e^{-E_a/RT} \quad (1)$$

where k' is $d(1/\eta_{\text{sp}})/dt$, A is the pre-exponential factor, R is the gas constant and T is the absolute temperature.

2.2.2. Small-strain oscillatory measurements

The small-strain oscillatory measurements were performed on a StressTech Rheometer from Reologica, Lund, Sweden. Measurements on FG were carried out on a plate and cone geometry ($d = 40$ mm, 4°). The temperature of the instrument was set to 4°C and 1.3 ml FG solution was applied.

Calanus crude extract (400 μl , 3 mg/ml *Calanus* crude protein) was added to 10 ml 10% (w/v) FG to a final concentration of 0.12 mg/ml at room temperature ($\sim 20^\circ\text{C}$) and stirred for 1 min before application to the rheometer. The temperature was kept constant at 4°C for 2 h before rapid heating up to 6, 8 or 10°C , followed by curing for 6 h at the ramping end temperature. The ramping of temperature was performed to increase the activity of the proteases, which is known to be low at 4°C [20], while still keeping the temperature below the melting temperature of the gelatin gel.

Storage modulus (G') was recorded and plotted as function of time and slopes (dG'/dt) were calculated by linear regression with $R^2 \geq 0.97$. The initial slope ($t_0 = 0$ h) was estimated by calculating the slope of the G' -curve in the linear region $t = 0-0.1$ h (first 6 min). The slope before temperature ramping was determined from the linear region of the G' -curve in the typical apparent steady state region at $t = 1.45-2$ h (15 min), whereas the slope after temperature ramping was determined from the linear region of the decreasing G' -curve at $t = 2-2.14$ h (8 min). The terminal slope was determined from the linear G' -curve at $t = 7-8$ h (60 min).

The storage modulus (G') for depolymerised FG (10% (w/v) in 50 mM Tris-HCl, pH 7.5) was recorded for approx. 8 h at constant 4°C . The storage modulus at infinite time, G'_{inf} , was estimated by plotting G' as function of time^{-1} and extrapolation to infinite time ($\text{time}^{-1} = 0$) [1].

2.2.3. Size exclusion multi-angle laser light scattering

The weight-average molecular weights of the FG samples were measured by size exclusion chromatography combined with multi-angle laser light scattering equipment (SEC MALLS—TSK pre-column β 4000 PWXL-DAWN DSP/Optilab DSP). The eluting buffer consisted of 0.05 M Na_2SO_4 and 0.01 M Na_2EDTA and the pH was 9. All samples and the eluting buffers were filtered prior to analysis to remove dust.

3. Results and discussion

3.1. Proteolytic degradation of fish gelatin in solution

The degradation of FG molecules in 1% (w/v) FG solutions by crude proteases from *C. finmarchicus* was measured by capillary viscometry [23]. Fig. 1 shows that the FG molecules were degraded by *Calanus* proteases since $1/\eta_{sp}$ is increasing with time. Depolymerisation of FG by proteases is not unexpected since the FG molecules at these temperatures exist as random coils in solution, totally lacking the protective triple helical structure found in collagen [18]. Hence, the random coil α -chains were readily exposed to proteolytic attacks causing a reduction in the average molecular weight and changing the molecular weight distribution. The reduced degree of polymerisation of the FG molecules led to a reduction in the specific viscosity. The decline in specific viscosity suggests that the crude extract contained endo-proteases, since internal proteolysis in the protein chains is required to give a depolymerisation leading to a reduction in specific viscosity.

In addition, Fig. 1 also shows that the degradation rate increased with temperature, as expected. A destabilisation (denaturation) of the proteases seemed to occur at temperatures $\geq 50^\circ\text{C}$, since the rate of degradation decreased with time at 50°C . At 40°C , the proteases were more stable as a function of reaction time, indicating that this temperature was closer to the overall temperature optimum for the proteases from *C. finmarchicus*. Although experimental limitations rendered viscosity measurements below 20°C impossible, it has earlier been shown by using haemoglobin as proteolytic substrate that the activity of this crude *Calanus* protease extract exhibited a specific activity 5–10 times lower in the 4– 10°C range compared to 40°C [20].

The Arrhenius activation energy (E_a) of the crude proteases was estimated [24] to be approximately 50.4 kJ/mol, based on the data for 20 – 40°C in Fig. 1 (see Section 2.2.1). This was similar to values (20.5–56 kJ/mol) for proteases from Krill (*Euphausia superba*) previously published Osnes et al. [24]. However, the estimated activation energy in this study reflected the average of all proteases present in the crude extract, whereas the values published by Osnes and co-workers refer to purified proteases.

3.2. Effect of proteases from *C. finmarchicus* on the dynamic storage modulus

As seen from Fig. 1, proteases broke peptide bonds in random coil FG, and this is expected to have a pronounced effect on the dynamic storage modulus (G') in FG gels assuming protease attacks

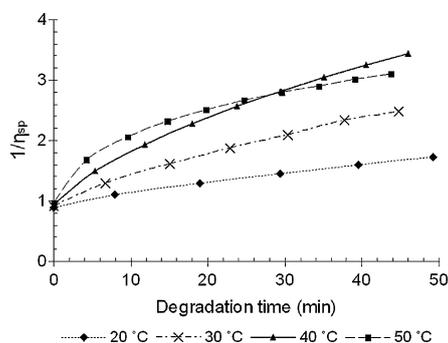


Fig. 1. Degradation of 1% (w/v) FG (0.067 mg/ml *Calanus* protein, 50 mM Tris-HCl, pH 7.5) measured by capillary viscometry at 20, 30, 40 and 50°C .

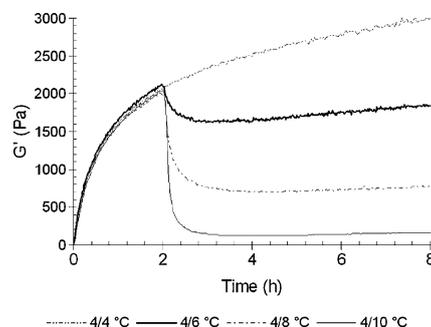


Fig. 2. Evolution of the dynamic storage modulus (G') upon time after curing at 4°C (2 h) and after subsequent ramping to 6, 8 and 10°C for 10% (w/v) FG (50 mM Tris-HCl, pH 7.5).

within the elastic segments. The storage moduli for 10% FG at four different temperatures were measured without and with crude proteases and the results are presented in Figs. 2 and 3, respectively. When a FG sample without proteases was applied and matured at 4°C , there was, as expected, a monotonic increase in G' without reaching an equilibrium value, which is typical for gelatin gels (Fig. 2) [10,25]. However, an immediate reduction in storage modulus was observed after ramping the temperature up to 6, 8, or 10°C after 2 h of curing at 4°C . It can be seen from Fig. 2 that the decrease in G' post ramping was followed by a gradual increment in the storage modulus towards the terminal holding time, suggesting a slow recovery or reorganisation of the triple helical structures in the gelatin gel network. It is well known that the kinetics and history of a gelatin gel influence both the build-up of the gel network as well as the mechanical strength of the final gel [10,25], which can also be seen from Fig. 2. The gelling temperature of 10% FG is close to 4°C , but the coil-to-helix temperature for the α -chains in solution has previously been reported to be well above the gelling temperature [7]. This implies that provided the right conditions and pre-history, gelatin may supply mechanical stability also at temperatures above the gelling temperature.

Fig. 3 shows the effect of temperature changes and addition of *Calanus* proteases to the FG system. The effect of proteolytic action was obvious, as can be seen by comparing Figs. 2 and 3. In the presence of proteases, an effect of both temperature and proteolysis on the storage modulus was observed. A comparison of Figs. 2 and 3 demonstrates that the absolute values of the storage moduli after curing at 4°C for 2 h were considerably lower for the systems with

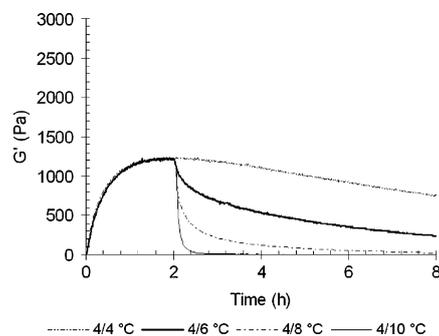


Fig. 3. Evolution of the dynamic storage modulus (G') upon time after curing at 4°C (2 h) and after subsequent ramping to 6, 8 and 10°C for 10% (w/v) FG (50 mM Tris-HCl, pH 7.5) in the presence of proteases (0.12 mg/ml *Calanus* crude protein).

proteases. This was most likely due to proteolytic cleavage within the elastic segments during curing at 4 °C. A slight depolymerisation may however, also have occurred as a result of proteolytic action during mixing at room temperature prior to the rheological analysis. The storage modulus of the samples kept at 4 °C (Fig. 3) exhibited a slope close to zero or below after approximately 2 h resulting from a weakening of the gel network suggesting that proteolysis also took place at 4 °C. It is reasonable to anticipate that interruption and damages, like, e.g. minor depolymerisation of molecules, will influence the sensitive initial formation of a gelatin gel network and thereby also the mechanical properties of these networks as a function of time. This may explain that even though the activity of the *Calanus* proteases was low at 4 °C [20], it may be sufficient to cause a reduced elasticity.

After ramping to higher temperatures (6, 8 and 10 °C) in the presence of proteases, a more rapid decrease in G' as well as an overall more profound reduction in gel elasticity was observed. As already referred to, it is fair to anticipate that these effects were caused by the sum of two factors, proteolytic action and thermal destabilisation of the gel network.

In order to distinguish between the thermal and proteolytic effects, the slopes (dG'/dt) were estimated in defined time intervals (see Section 2.2.2) of the setting curves presented in Figs. 2 and 3. The initial slopes are comparable for all samples without or with proteases (Fig. 4a and b). The negative slopes after temperature ramping did however increase with increasing temperatures, as can be seen from both Fig. 4a and b. In Figs. 3 and 4b it can be seen that the decrease in G' as a function of time without ramping (at constant 4 °C) was lowest and only due to proteolytic activ-

ity. The slopes of the G' curves immediately following temperature adjustment were mainly influenced by the increase in temperature. However, by comparing Figs. 2 and 3 it can be seen that the G' curves after ramping exhibit different trends. All slopes for the G' curves at the end of the 8-h period in Fig. 4b are negative and as discussed above, most probably due to proteolysis. The results for FG without proteases (Figs. 2 and 4a) exhibited, as already mentioned, low but positive slopes suggesting a slow recovery of the network structure. The samples with proteases, however, all exhibited negative slopes, which can be attributed to the proteolytic activity, and also that the proteolysis overshadowed the percolation processes observed in the samples without proteases.

The absolute values of the slopes shown in Fig. 4a are generally larger than those in Fig. 4b, which indicates that the relative changes in storage modulus (dG'/dt) were larger in absence of proteases. The main difference, however, occurred after ramping and at the end of the curing time. The sample at constant 4 °C (Fig. 4a) exhibited a positive slope over the entire measuring period (8 h), and all samples exhibited positive slopes towards the end of the holding period independent upon the ramping temperature. However, the slopes decreased with increasing holding temperature, which was expected since the recovery of the gel network should occur faster at lower temperature in the absence of degrading agents.

Fig. 4b shows that dG'/dt towards the terminal curing time decreased with increasing temperature, which apparently may seem unlikely. A possible explanation may be that the higher the holding temperature (e.g. 8 °C), the less coherent the network will be due to a partial unwinding of junction zones. It is therefore more likely that the proteolytic cleavage at 4 °C will occur within an active elastic segment leading to a more profound effect on the dynamic storage modulus.

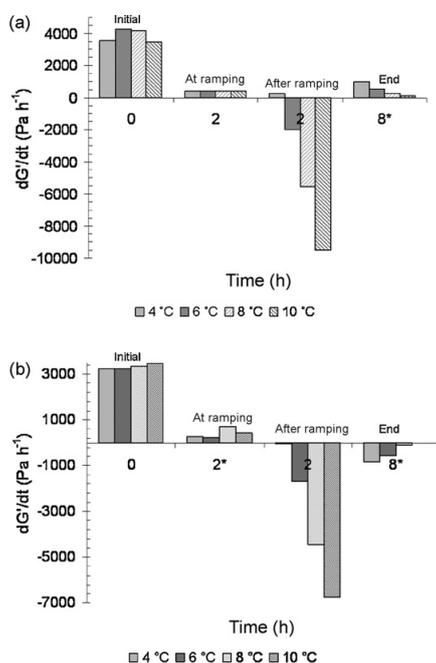


Fig. 4. (a) Slope (dG'/dt) as function of time at 4, 6, 8 and 10 °C for 10% (w/v) FG at pH 7.5 without proteases. The slopes were calculated by linear regression as described in Section 2.2.2. *Values multiplied with 10. (b). Slope (dG'/dt) as function of time at 4, 6, 8 and 10 °C for 10% (w/v) FG at pH 7.5 with protease (0.12 mg/ml *Calanus* crude protein). The slopes were calculated by linear regression as described in Section 2.2.2. *Values multiplied with 10.

3.3. Storage modulus at infinite time for fish gelatin as function of molecular weight

Since the gelling of gelatin is a non-equilibrium process [25], the storage modulus is expected to increase over time at the annealing temperature. Fig. 5a shows the dynamic storage modulus of FG of different weight-average molecular weights as function of time at 4 °C. A lower storage modulus with decreasing molecular weight was observed. However, in order to compare the terminal moduli (infinite curing time) of the different molecular weights, the data between 5 and 6 h (Fig. 5a) were extrapolated to infinite time by plotting G' against time^{-1} , and the result is shown in Fig. 5b. The lowest molecular weight (28 kDa) did not go through a sol to gel transition and exhibited a very low G' -value (Fig. 5b).

Fig. 5b shows the storage moduli extrapolated to infinite time, G'_{inf} , for FG samples of different weight-average molecular weights after curing at 4 °C. The results showed a rapid decrease in storage modulus as the molecular weight decreased below 50 kDa. This suggests that FG below 50 kDa was less capable of creating a continuous gel network at 4 °C and at a concentration of 10%, most probably due to shorter chain lengths preventing percolation and increasing the loose end and sol fraction. It has previously been reported that the length of a stable triple helical junction zone in a gelatin gel is between 20 and 30 amino acids [15]. By using 110 g/mol as an average molecular weight for the amino acids in gelatin, the findings by Harrington suggest that the “molecular weight” of an α -chain participating in a stable triple helical junction zone should be at least 2200–3300 g/mol. It is however not sufficient that an α -chain participates in only one stable triple helical junction zone to form a three dimensional gel network, i.e. a higher degree of functionality is required, and the junction zones must be interconnected by elastic segments. All together, this implies that the molecular weight of gelatin molecules contributing to the

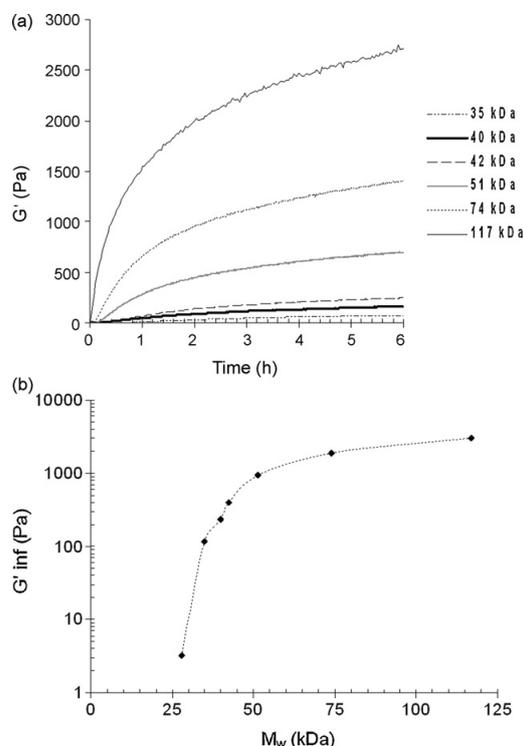


Fig. 5. (a) Evolution of the dynamic storage modulus (G') upon time at 4°C for 10% (w/v) acid hydrolysed FG samples (50 mM Tris-HCl, pH 7.5). (b) The infinite storage modulus (G'_{inf}) of 10% (w/v) FG as function of M_w (50 mM Tris-HCl, pH 7.5).

mechanical stability of a gelatin gel must be considerably higher than 3300 g/mol.

The data in Fig. 5b suggests that the acid hydrolysis led to an increase in the fraction of loose ends and therefore a reduction in gel strength due to a reduced number of elastically active segments. It is generally known that an increased fraction of loose ends and soluble molecules as obtained by reducing the M_w will reduce the mechanical properties of the gel [26]. Taking the physical gelatin network into consideration, i.e. junction zones of a certain size and extension rather than point-like junctions, the rapid reduction in G' below 50 kDa may partly also be due to scissions in the pyrrolidine-rich, junction forming regions. In turn, such a shortening of potential junction forming entities will eventually result in lengths of these sequences below the critical size for stable junction zone formation (at least 20–30 amino acids), implying fewer junction zones and elastic segments per unit volume of the gel. All in all, a reduced number of elastically active segments as well as a reduced number of stable junction zones (reduced functionality) will drastically lower the mechanical properties of the final gel as the molecular weight is reduced.

As can be seen from Fig. 3, the storage modulus for the sample at constant 4°C and in the presence of proteases was reduced to 1000 Pa after approximately 4.6 h, and the data presented in Fig. 5 therefore suggest that the average molecular weight of the proteolytically degraded FG could be close to 50 kDa at this point. It should, however, be kept in mind that some degree of hysteresis is expected in these systems implying that there may be a difference between studying the gel formation with an already depolymerised

gelatin sample and studying depolymerisation of a pre-formed gelatin gel. For example, no rapid reduction in G' was observed after 4.6 h (Fig. 3), which could suggest that the proteases do not attack the pyrrolidine-rich regions, which are in the ordered conformation in the gel, in any substantial way.

4. Conclusions

The presented results offer new insights into the relationship between the rheological properties of cold-water FG and the effect of degradation by proteases.

The reduction in mechanical stability of FG gels in the temperature range of 4–10°C in the presence of proteases may make it difficult to apply FG for encapsulation of protease-containing zooplankton such as *C. finmarchicus*. If FG, or any type of gelatin, is to be used as the gel-forming matrix in the presence of proteases, it may be wise to inactivate the enzymes prior to encapsulation. Such an inhibition is most easily achieved by an elevated temperature treatment, but this may detract from the nutritional properties of the final product. It may therefore be concluded that at present, FG is not suitable for the encapsulation of protease-containing bioproducts, and that other matrix forming polymers not susceptible to protease activity, such as polysaccharides, should be preferred.

Acknowledgements

Senior engineer Ann-Sissel Ulset should be thanked for helping with SEC-MALS. Engineer Ingrid A. Draget is also thanked for support, advice and practical help. This work was supported by the Norwegian Research Council through the Strategic University Program “CODTECH—a process oriented approach to intensive production of marine juveniles with main emphasis on cod” (project no.153422). Members of the Calanus project (CALANUS—exploitation of zooplankton as bioresource for fish feed and industrial raw material—a complete value chain evaluation, project no. 143184), Nils Tokle and Nils Melhuus are thanked for giving us the opportunity to catch Calanus in the spring 2006 at Hitra/Frøya, Norway.

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Paper IV

**Release of a macromolecular component from
chitosan-coated alginate capsules; effect of F_A and
lysozyme**

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Abstract

Ca-alginate capsules coated with chitosan exhibit a reduced release of encapsulated blue dextran. A concentration of 0.1 mg/ml chitosan in the gelling bath was sufficient to reduce this release significantly for chitosans with $F_A = 0.12$ and 0.4. The release was affected by the degree of acetylation of the chitosan and was found to decrease with decreasing F_A . The presence of lysozyme had a pronounced effect on the release for capsules with highly deacetylated chitosan ($F_A = 0.12$). The release was significantly reduced probably due to non-productive binding of lysozyme to chitosan and thereby increasing the density of the coating layer. The presence of lysozyme increased the release of BD in the case with $F_A = 0.4$, which suggested degradation of the chitosan layer. This study has focused on the importance of the degree of acetylation of the chitosan with regard to the stability of chitosan-alginate capsules.

Keyword: Alginate; Chitosan; Blue dextran release; Degree of acetylation; Lysozyme

1. Introduction

Alginate, an extensively used matrix provider for encapsulation, is a polysaccharide composed of $\beta(1\rightarrow4)$ -D-mannuronic acid (M) and $\alpha(1\rightarrow4)$ -L-guluronic acid (G) and is mainly isolated from brown algae (Draget et al. 2006). This polysaccharide yields temperature stable gels in the presence of some divalent metal ions (e.g. Ca^{2+} or Ba^{2+}). The gel strength is dependent on G content (F_G) and the sequence of these residues (G-block length). Alginate is widely used as a thickening agent in food and in immobilization of enzymes and cells (Draget et al. 2006), and is in this paper used as encapsulation matrix.

Chitosan is a linear polysaccharide composed of randomly distributed $\beta(1\rightarrow4)$ -linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (Vårum et al. 2006). It has a number of commercial and possible biomedical uses due to its bioadhesive properties. Chitosan is produced commercially by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (crabs, shrimp, etc.). The degree of acetylation (F_A) can be determined by NMR spectroscopy (Vårum et al. 1991). The amino groups in chitosan have a pK_a value of ~ 6.5 , which means that chitosan is positively charged at low pH and will have the capability to attach to negatively charged surfaces. Chitosan may bind to e.g. alginate capsules at the appropriate pH, since alginate is negatively charged at pH above the pK_a of its carboxyl groups (~ 3.5). The binding of chitosans to alginate beads has been studied by others (Gåserød et al. 1999; Ribeiro et al. 1999; Silva et al. 2005) using different techniques for capsule formation, such as bead generators and emulsion technology. Generally, using bead generator, the coating is achieved by either a one-stage or a two-stage procedure. In the one-stage procedure, coated capsules are produced by adding the alginate solution directly into a gelling solution (with Ca^{2+}) containing chitosan. In the two-stage procedure, the alginate capsules are left to harden for 15-30 min before they are introduced to a solution containing chitosan.

Chitosan-alginate capsules are considered stable and release of the immobilized macromolecules is effectively retarded by the chitosan. However, these capsules would be unstable in e.g. the GI tract due to the presence of lysozyme, which is known to degrade chitosan depending largely on the degree of acetylation (Vårum et al. 2006).

Lysozyme is an enzyme (EC 3.2.1.17) that breaks down bacterial cell walls by hydrolysis of 1,4- β -linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan and between N-acetyl-D-glucosamine residues in chitin and chitosan (Vårum et al. 2006). It is abundant in a number of secretions, such as tears, saliva and mucus. It is also present in hen egg white (HEW).

The hydrolytic activity and the mechanism of lysozyme has been extensively studied (Vårum et al. 2006). E.g. the activity of lysozyme is known to be highly affected by F_A (degree of acetylation), and experiments have suggested that the degradation rate of chitosan is proportional to F_A in the fourth power. In addition, NMR studies have shown that lysozyme have an active binding site consisting of six subsites, commonly denoted A-F where scission occurs between subsite D and E. Degradation studies and NMR determination of the identities have suggested that 4 N-acetylated residues has to be contained in the lysozyme binding site to obtain maximum initial degradation rate. However, the binding of lysozyme to partially and highly N-actylated chitosans have been studied and it has been found that N-acetylated units (monomer) surrounded by deacetylated units bind preferentially in subsite C, without depolymerization of the chitosan chain (Kristiansen et al. 1996; Kristiansen et al. 1998).

Coating of feed capsules with chitosan could be useful way of controlling the content of immobilized macromolecules such as proteins. However, if these capsules were to be taken orally, they would have to be readily digestible. Thus, the capsules must be degraded by enzymes such as lysozyme, and the ideal situation would be to coat the feed capsules with chitosan with optimal F_A , which would stabilize the immobilized molecules during production and storage, but also be readily degraded during digestion.

The scope of the present study was to investigate the effect of chitosan-coating on the release of an immobilized macromolecule from chitosan-alginate capsules, and the effect of lysozyme activity. In this study, blue dextran (BD) with an average molecular weight of 2000 kDa was chosen as model substance because it does not carry any electrostatic charges and should thus not affect the binding between chitosan and alginate, and could hence be considered an inert polymer. Dextran can also be considered to be a biopolymer with a highly compact configuration in aqueous solutions due to its flexible $\alpha(1\rightarrow6)$ -linked backbone structure (Tirtaatmadja et al. 2001).

2. Materials and Methods

2.1. Materials

The sodium alginate was a gift from FMC Biopolymer. This alginate was isolated from the stipe of *Laminaria hyperborea* and has an intrinsic viscosity $[\eta]$ of 980 ml/g and guluronate content of 64 % ($F_G=0.64$). The chitosans were a kind gift from Professor K.M. Vårum (see Table 1). Blue dextran 2000 (BD) was procured from Pharmacia Fine Chemicals. The hen egg white (HEW) lysozyme (L6876) was purchased from Sigma-Aldrich. Sodium acetate (pro analysi) and calcium chloride dihydrate (pro analysi) were procured from Merck. All other chemicals were of analytic grade.

Table 1. Some properties of the chitosans used in this study.

Chitosan	F_A	$[\eta]$ (ml/g)	M_w (kDa)*	Comment
1	0.01	610	191	Dissolved in 50 mM NaAc/ 100 mM $CaCl_2$
2	0.12	846	273	Dissolved in 50 mM NaAc/ 100 mM $CaCl_2$
3	0.35	760	243	Dissolved in 0.1 M HAc
4	0.40	800	257	Dissolved in 50 mM NaAc/ 100 mM $CaCl_2$
5	0.49	900	292	Dissolved in 0.1 M HAc

*approximated with the MHS-equation, $[\eta]=8.43 \times 10^{-3} M_w^{0.93}$ (Berth et al. 2002)

The chitosans were dissolved directly in 50 mM NaAc/ 100 mM $CaCl_2$ pH 5 or with double concentration in 0.1 M HAc (see table 1). Chitosans in 0.1 M HAc were titrated to pH 5 with NaOH and the volume was adjusted with deionized water to give 50 mM NaAc and correct chitosan concentration.

2.2. Capsule formation (one-stage method)

All alginate and blue dextran solutions were made by dissolving the components in 50 mM NaAc, pH 5. 3.6 % alginate was mixed with equal amount of 2 % BD to give an Alginate-BD solution with 1.8 % alginate and 1 % DB, and the final solution was filtered through a 5 μ m membrane filter. Alginate-BD capsules were produced by the aid of an electrostatic bead generator with an electrostatic potential of 7 kV (Gåserød

et al. 1999). 3 ml alginate-BD solution was pumped through a 0.35 mm needle into 100 ml gelling solution of 50 mM NaAc and 100 mM CaCl₂ and different concentrations of chitosan. The distance between the tip of the needle and the gelling solution was 30 mm and the flow rate was kept at 10 ml/h. The pH of the gelling solution was kept at 5 in all experiments to optimize the potential electrostatic interaction between alginate and chitosan. The release of BD was monitored by measuring the absorbance at 620 nm as function of time. The capsules were kept in the gelling solution with mild stirring and samples were taken after 5 min, 30 min, 1, 2, 3 and 5 hours.

The capsules were coated with chitosan with F_A (degree of acetylation) varying from 0.01 to 0.49 (see table 1, section 2.1). Concentrations of chitosan in the gelling solution varied from 0 to 0.4 mg/ml. The chitosan concentration was kept a constant 0.2 mg/ml in the experiments with varying F_A and lysozyme treatment (section 3.2 and 3.3)

2.3. Lysozyme treatment

Chitosan coated capsules were cured for 45 min and washed 3 times with 40 ml 50 mM NaAc/10 mM CaCl₂, pH 5. The washed capsules were dispersed in 40 ml 50 mM NaAc/10 mM CaCl₂ pH 5 with 0.6 mg/ml HEW lysozyme. The washing procedure lasted approx 15 min. A control without lysozyme was investigated for comparison. The capsules/solutions were kept in 100 ml glass beakers, and were incubated with mild stirring at ambient temperature (~23 °C). Release of BD was monitored by measuring the absorbance in the surrounding buffer at 620 nm as function of time.

3. Results and Discussion

3.1. *BD release as function of chitosan concentration*

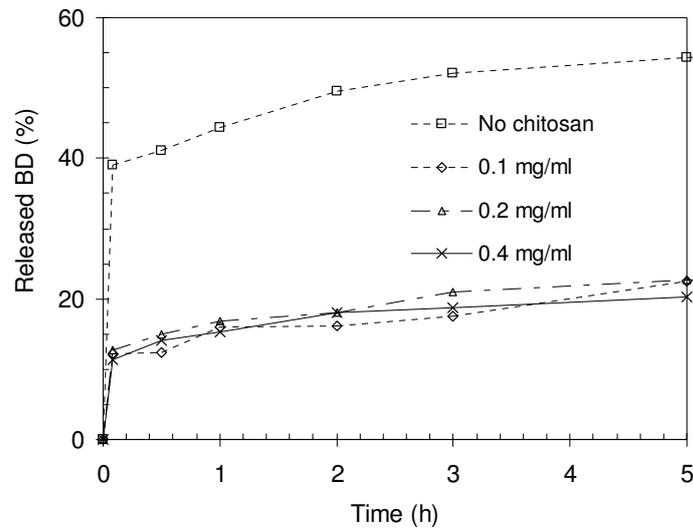


Fig. 1 Release of Blue dextran from chitosan coated alginate capsules as function of chitosan concentration in the gelling bath ($F_A=0.12$). Buffer was 50 mM NaAc/100 mM CaCl_2 , pH 5.

Fig. 1 shows the release of BD from alginate capsules coated with chitosan with a $F_A=0.12$. In the case of no coating, the initial release was significant (40 %) and it continued to increase with time, reaching approximately 54 % after 5 hours. The introduction of chitosan led to a significant reduction in BD release, which suggests that the capsules have been effectively coated and that diffusion of BD out of the alginate matrix has been obstructed by the presence of chitosan on the surface. In addition, there seemed to be no concentration dependence above 0.1 mg/ml chitosan in the gelling bath, which suggests that the surface of the alginate capsules has been saturated with chitosan already at 0.1 mg/ml chitosan. BD is a large biomolecule with an average molecular weight of approximately 2 million Daltons, and is expected to have a slow release from such particles. It would therefore be reasonable to suggest

that only a small amount of chitosan would be needed to reduce the release of such large molecules.

High G-alginates create open networks, which allow fairly large molecules to diffuse easily out of such a network. Martinsen et al. (1992) found that a molecular weight of more than 300 kDa was needed for globular protein molecules to exhibit diffusion constants lower than that of water. Even if BD has a significant higher molecular weight, it may still diffuse out because dextrans have a highly compact conformation (Tirtaatmadja et al. 2001).

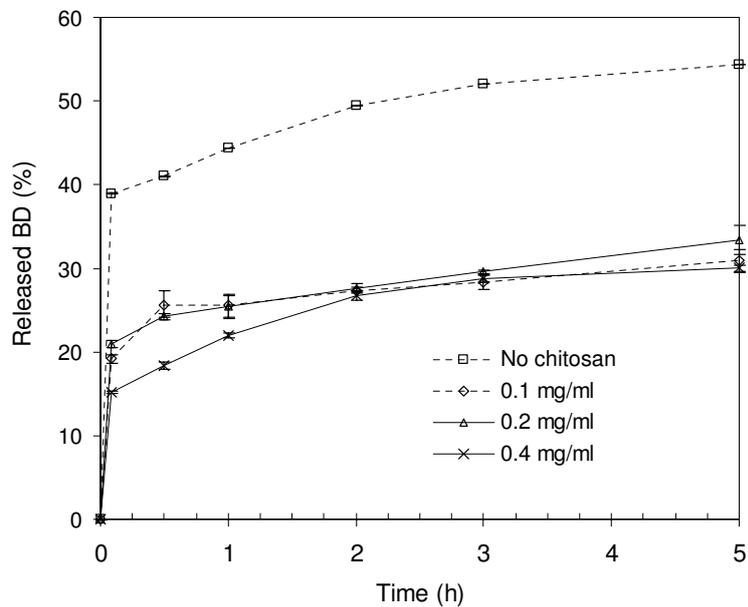


Fig. 2 Release of Blue dextran from chitosan coated alginate capsules at as function of chitosan concentration in the gelling bath ($F_A=0.40$). Buffer was 50 mM NaAc/100 mM $CaCl_2$, pH 5.

Fig. 2 shows the release from capsules coated with chitosan with $F_A=0.4$ at three different concentrations in the gelling bath. No significant difference was observed for 0.1 and 0.2 mg/ml chitosan. At 0.4 mg/ml however, there seemed to be a significant lower release the 2 first hours. After 2 hours, the rate of release was similar to the 2 lower concentrations, suggesting that higher concentration of chitosan only affects the initial release of BD and does not have any effect on longer storage times.

3.2. BD release as function of F_A

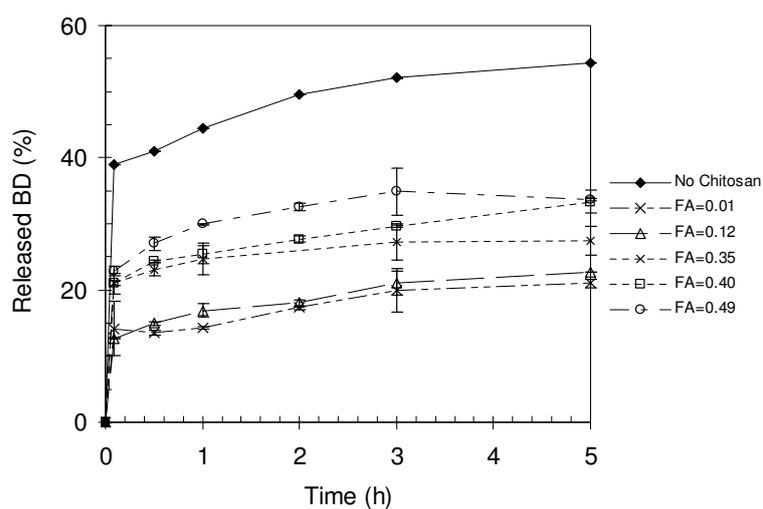


Fig. 3 Release of blue dextran as function of F_A . The buffer was 50 mM Acetate/100 mM CaCl_2 , pH 5. The chitosan concentration in the gelling bath was 0.2 mg/ml.

Fig. 3 shows the release of BD as function of F_A . The release was lowest for capsules coated with the least acetylated chitosan ($F_A = 0.01$), as expected. This chitosan coating led to approximately 60 % reduction in the release of blue dextran after 5 hours. However, the release with $F_A=0.01$ was not significantly lower than that of capsules with chitosan with $F_A=0.12$, suggesting that a degree of acetylation of 0.12 is sufficient for effective coating. Chitosan with $F_A=0.4$ and 0.49 led only to approximately 35-40 % reduction in BD release after 5 hours. This release is significantly higher, which suggests that chitosans with higher degree of acetylation are less effective as coaters due to a lesser amount of positive charges on the chitosan molecule as well as containing more bulky acetyl groups that could prevent close packing of the capsule.

3.3. Effect of lysozyme on BD release

The effect of lysozyme on washed chitosan coated capsules was investigated for chitosans with F_A of 0.12 and 0.40. The concentration of chitosan in the gelling bath was kept constant at 0.2 mg/ml.

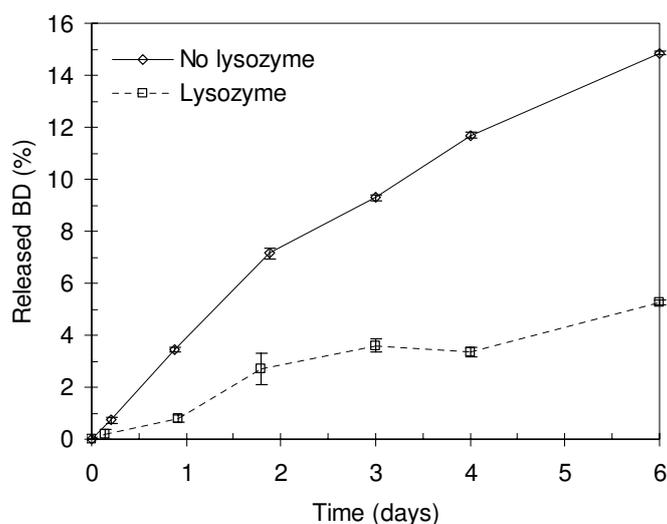


Fig. 4 Effect of HEW lysozyme on chitosan coated and washed alginate capsules ($F_A=0.12$). Buffer was 50 mM NaAc/10 mM CaCl_2 , pH 5. The lysozyme concentration was 0.6 mg/ml.

Fig. 4 shows the effect of lysozyme on coating with $F_A=0.12$. The release was significantly inhibited by the presence of lysozyme, which at first sight may seem surprising. However, lysozyme is known to attach strongly to chitosans with a high fraction of mono-acetylated monomers without subsequent hydrolysis (non-productive binding). It is therefore reasonable to suspect that the release is reduced due to the formation of a lysozyme-chitosan layer on the surface of the alginate capsules. In addition, HEW lysozyme is also known to be a small, globular peptide (~14 kDa) which should be able to diffuse easily into the alginate capsule and saturate the network. At pH 5, the HEW lysozyme molecule has a high net positive charge (pI 11.35), and should be able to bind to the negatively charged carboxyl groups on the

alginate. All in all, these effects would greatly obstruct the release of dextran leading to a reduced release.

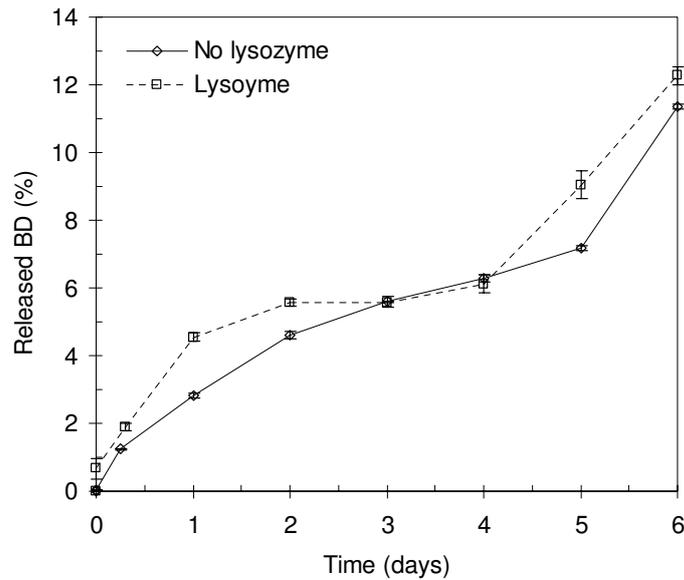


Fig. 5 Effect of HEW lysozyme of chitosan coated and washed alginate capsules ($F_A=0.4$). Buffer was 50 mM NaAc/10 mM CaCl_2 , pH 5. The lysozyme concentration was 0.6 mg/ml.

Fig. 5 shows the effect of lysozyme for $F_A=0.4$. The presence of lysozyme led to an increased release the first 2 days, followed by a 2 day lag period before a new period of release on the following 3 last days of incubation. The release of BD seemed to be affected by lysozyme; however, the non-linear behavior was not as expected. This suggests that lysozyme is degrading the chitosan layer leading to an initial higher release of BD. As mentioned above, HEW lysozyme has a net positive charge and may therefore interact electrostatically with the alginate, which again may have slowed the hydrolysis of chitosan and led to lysozyme saturated capsules.

By comparing Fig. 4 and Fig. 5, it can be seen that the total release of BD after 6 days are slightly higher in the case with $F_A=0.12$ than $F_A=0.4$ in the absence of lysozyme. This was surprising; however, one possible explanation may be the difference in BD content in the capsules after washing. Capsules with $F_A=0.12$

contained 0.85-0.86 % BD, whereas capsules with $F_A=0.4$ contained 0.78-79 %. Thus, capsules coated with chitosan with $F_A=0.12$ would have had a slightly higher concentration gradient between the interior of the capsules and the surrounding buffer. This driving force, though weak, may explain some of the difference between Fig. 4 and Fig. 5.

4. Conclusion

Ca-alginate capsules coated with chitosan exhibit a reduced release of encapsulated blue dextran. A concentration of 0.1 mg/ml chitosan in the gelling bath was sufficient to significantly reduce the release for chitosans with $F_A = 0.12$ and 0.4. The initial release was affected by the degree of acetylation of the chitosan, and the release of BD was found, as expected to decrease with decreasing F_A .

The presence of lysozyme had a pronounced effect on the release for capsules with highly deacetylated chitosan ($F_A=0.12$). The release was significantly reduced probably because of complex formation between the chitosan layer and lysozyme, which was a result of non-productive binding to mono-acetylated units.

In case of $F_A=0.4$, the presence of lysozyme initially increased the release of BD, which suggested degradation of the chitosan layer as suspected. However, the release exhibited a non-linear behavior, which was unexpected. There may have been electrostatic interactions between the net positively charged lysozyme molecules and the negatively charged alginate network, which led to this unexpected behavior.

This study shows that chitosan may be used to reduce the release of large, flexible biomacromolecules from alginate capsules. However, the choice of chitosan would highly affect the stability of such particles in respect to the amount and rate of release over time, and the degradability in the presence of chitosan degradable enzymes such as e.g. lysozyme. The most important parameter is the F_A of the chitosan. In feed applications, the choice of chitosan would be the one, which could prevent release of nutrients during manufacturing and storage, but be readily degraded in the digestive system. This study suggests that highly deacetylated chitosans would not be suitable in feed applications because of insufficient degradability, whereas moderately deacetylated chitosans would be more suited in such a system.

Acknowledgments

We thank Prof. K. M. Vårum for valuable discussions and for supplying the chitosan used in this paper. This work was supported by the Norwegian Research Council through the Strategic University Program “CODTECH – A process oriented approach to intensive production of marine juveniles with main emphasis on cod” (project no.153422). Dr. Yrr Mørch and Dr. Berit L. Strand should we thanked for assistance and help with the electrostatic bead generator.

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