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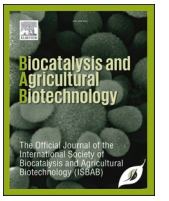

Calanus finmarchicus as a novel source of health-promoting bioactive peptides: Enzymatic protein hydrolysis, characterization, and *in vitro* bioactivity

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ABSTRACT

Calanus finmarchicus is a crustacean currently used as a source of marine lipid. The lipids are extracted by enzymatic protein hydrolysis, while the remaining peptide fraction is regarded as a byproduct. In the present work, ten different commercial proteases and endogenous *C. finmarchicus* proteases were used to produce a set of 63 protein hydrolysates. Protease concentration and hydrolysis time were varied. Hydrolysates were characterized using size-exclusion chromatography and ^{1}H nuclear magnetic resonance spectroscopy. Addition of commercial proteases had unremarkable effect on the yield and molecular weight distribution. This was attributed to the strong impact of endogenous enzymes dominating the hydrolysis process. However, multivariate classification based on ^{1}H NMR spectra revealed subtle variations in composition of hydrolysates produced using different enzymes. The hydrolysates were further evaluated for DPP-IV inhibition and antioxidant activity. The hydrolysates showed significantly higher bioactivity than the unhydrolyzed control. A representative hydrolysate (**CaFi55**) was fractionated using semipreparative size-exclusion chromatography. A fraction consisting of short peptides with an average chain length of five amino acids (**F2**), was identified as a major contributor to the DPP-IV inhibitory activity (IC_{50} = 0.70 ± 0.07 mg/mL).

1. Introduction

Many marine species represent vital sources of food ingredients. In addition, various bioactive compounds displaying a range of bioactivities have already been isolated from marine sources (Ennaas et al., 2015; Macedo et al., 2021; Reen et al., 2015; Teixeira-Guedes et al., 2023). Zooplankton *Calanus finmarchicus*, commonly named red feed, is a crustacean belonging to the copepod group. It is highly abundant in the Norwegian Sea and parts of the Arctic Ocean, and it is currently used as a source of marine lipids. Recent advances in harvesting and processing technologies have opened enormous possibilities for extraction and development of food and feed ingredients from *C. finmarchicus*. Lipids (predominantly wax esters), proteins and chitin are among the valuable constituents of *C. finmarchicus*, as well as the potent antioxidant astaxanthin which gives the animal a distinctive red color (Eysteinsson et al., 2018; Fraser et al., 1989; Schots et al., 2020). *C. finmarchicus* oil can be extracted using different processing technologies. Traditional method consists of cooking, while modern approach utilizes enzymatic protein hydrolysis (EPH). The latter is considered advantageous due to

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the improved efficiency of lipid extraction (Vang et al., 2013).

Enzymatic protein hydrolysis (EPH) is an expedient biotechnological tool that enables, in addition to the targeted oil extraction, recovery of proteins and peptides from various protein-rich biomasses. EPH utilizes proteases to degrade proteins from a given biomass, producing water-soluble peptides and single amino acids (Lindberg et al., 2021a). One of the virtues of EPH is the possibility of tuning processing conditions (e.g., enzyme choice, hydrolysis time and temperature) to obtain the final product with varying chemical and functional properties (Lima et al., 2019; Sorokina et al., 2022; Tavano, 2013). Currently, peptides recovered from EPH of *C. finmarchicus* are considered as a by-product of the oil extraction and are limited to feed applications. However, protein hydrolysates of *C. finmarchicus* constitute various small peptides and can serve as bioactive ingredients either in the form of nutraceuticals or functional foods.

Marine sources comprise a large variety of amino-acid sequences of linear and cyclic peptides, depsipeptides and non-natural amino-acids (Lazcano-Perez et al., 2012). A number of marine peptides have been proven to exhibit antioxidant, immunomodulatory and antidiabetic properties (Pavlicevic et al., 2020). Bioactive peptides, in general, can show bioactivity in their native form, or can be incorporated into protein structures of the source, and exhibit bioactivity when released (e.g. by protein hydrolysis). Small molecular weight peptides from protein hydrolysates (2–20 amino acids) can exert various health promoting effects such as blood glucose regulation, important for type 2 diabetes (T2D) management (Chakrabarti et al., 2018; Romero-Garay et al., 2022). In the present study, peptides recovered from EPH of *C. finmarchicus* were studied for potential antidiabetic and antioxidant effects.

The risks of T2D complication are mainly linked to an increase in hemoglobin A1c (HbA1c) (Ray et al., 2009). Hence, control of blood glucose level (A1C) constitutes one of the major therapeutic strategies in management of T2D. Incretins (glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1)) are vital elements in regulation of blood glucose levels, responsible for up to 60% of the insulin response to a given glucose load (Neumiller et al., 2010). A serine protease enzyme, dipeptidyl peptidase IV (DPP-IV), degrades GLP-1 and GIP and drastically reduces the half-lives of these important hormones. Hence, selective DPP-IV inhibitors can prolong the vital role of incretins in glucose homeostasis. Alternative to pharmaceuticals, bioactive peptides have been proposed as a substitute for DPP-IV inhibitors that can be used for management of HbA1c in the form of nutraceuticals or functional foods (Ray et al., 2009).

In addition, many peptides have shown the potential to alleviate the effects of oxidative stress (Sarmadi and Ismail, 2010). Oxidative stress can cause abnormalities in the structures of enzymes or cells as well as damage on mitochondrial level, and it has been linked to multitude of diseases (Sharifi-Rad et al., 2020). Oxidants in the organism originate both from exogenous sources, such as food or environmental pollutants, and from endogenous sources, as they are likewise a product of mitochondrial activity (Esfandi et al., 2019). It is also known that antioxidants help prolong the shelf life of food, especially when it contains unsaturated fats (Barouh et al., 2022).

In the present study, a set of 63 hydrolysates was produced from *C. finmarchicus* biomass, using 10 different commercial proteases and endogenous proteases of *C. finmarchicus*. Protease concentration and hydrolysis time were varied. The hydrolysates were characterized by size-exclusion chromatography and ^{1}H nuclear magnetic resonance spectroscopy; and screened for antioxidant activity and DPP-IV inhibition. To the best of the authors knowledge, the present work is the first comprehensive study of *C. finmarchicus* protein hydrolysates and their potential as DPP-IV inhibitors.

2. Materials and methods

2.1. Materials

Flavorpro 786 MDP, Flavorpro 766 MDP, Promod D24 MDP and Flavorpro Umami 852 MDP were obtained from Biocatalysts Ltd. (Cardiff, UK). Endocut 01 and Endocut 04 L were obtained from Tailorzyme ApS (Herlev, Denmark). Alcalase and Flavourzyme were obtained from Sigma-Aldrich (St. Louis, MO, USA). Corolase 2 TS was obtained from AB Enzymes GmbH (Darmstadt, Germany). Bromelain was obtained from Gunung Sewu (Jakarta, Indonesia). The formulations and amounts of proteases in respective protease preparations are given in Table S1 in the Supplementary information (SI). Chemicals used for ABTS decolorization assay (2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), ammonium persulfate and (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dipeptidyl peptidase IV (DPP-IV) Inhibitor Screening Assay Kit (ab133081) was purchased from Abcam PLC (Cambridge, UK). *Calanus finmarchicus* biomass was obtained from Zooca™ - Calanus AS, Norway. HPLC-grade acetonitrile, trifluoroacetic acid, monosodium phosphate and molecular weight standards used for the SEC analysis (see Table S2 in the SI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The water used for the HPLC mobile phase, sample preparation and for calibrating the refractometer was purified by deionization and 0.22 μm membrane filtration (MilliporeSigma, Burlington, MA, USA).

2.2. Enzymatic hydrolysis

The biomass (*C. finmarchicus*) used as a substrate in hydrolysis reaction was stored at −40 °C. Before hydrolysis, it was partially thawed by keeping it over night at 4 °C and homogenized using a standard kitchen blender (Xplode Vital blender BBLSP 1800S, Wilfa, Oslo, Norway) for approximately 30 s, with added distilled water to aid the process. Homogenized mixture was transferred to a reactor and was suspended in additional distilled water, keeping a final ratio of 1:1 w:w/water:biomass. The enzymatic hydrolysis reactions were performed in a heated Reactor-Ready™ jacketed reaction vessel (Radleys, Saffron Walden, UK). Heating of reaction vessel was achieved by circulating water through the system using a circulator pump (Julabo GmbH, Seelbach, Germany). When the reaction mixture reached 50 ± 1 °C (approximately 45 min), a selected protease was added to the mixture to start the hydrolysis reaction. To define the hydrolysis conditions, a test hydrolysis with Alcalase (0.5% w:w/protease:biomass) was performed, the samples from the

reaction mixture were taken at ten time-points (0.5.2.5, 5, 10, 15, 20, 30, 40, 50 and 60 min) and analyzed by size-exclusion chromatography (Fig. S1 and Table S3, SI). To produce hydrolysates **CaFi01–CaFi60**, ratio of the protease and the biomass was either 0.1%, 0.5% or 1.0% w:w/protease:biomass, and the reaction time was either 30 min or 60 min for the different batches. Overview of reaction conditions is presented in Table 1. After the appropriate reaction time, hydrolysis was quenched by thermal inactivation of the protease. Thermal inactivation was achieved by rapid heating (1.5 min) of the reaction mixture in the microwave oven (Menumaster, ACP, IA, USA), after which the mixture was kept at 95 °C inside the water bath (Precision GP 10, Thermo Fisher Scientific, Waltham, MA, USA) for additional 15 min. Batches with endogenous enzyme hydrolysis (**CaFi61** and **CaFi62**) were prepared as described above but without addition of proteases. To prepare the unhydrolyzed control batch (**CaFi63**) biomass was partially thawed by keeping it over night at 4 °C, homogenized, and then submitted directly to the deactivation protocol. After inactivation, the mixture was centrifugated for 45 min, at 5346 *g* and 40 °C, using Multifuge 4 KR centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) to separate the sediment from the liquid phase. The supernatant was collected and filtered using Pall® Depth Filter Sheets T2600. A sample of each hydrolysate (1 mL) was taken from the water phase, filtered through a Millex-HV PVDF 0.45 μm 33 mm filter (MilliporeSigma, Burlington, MA, USA) to determine the Brix value. Brix values were measured on 100 μL of hydrolysates using a Reichert AS200 Digital Handheld Refractometer (Ametek Reichert Technologies, NY, USA). The refractometer was calibrated against 100 μL of mQ water for every 4–6 samples. The rest of the water phase was aliquoted in 250 mL plastic containers and stored frozen at −40 °C until lyophilized using a Gamma 1–16 LSC plus freeze dryer (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany).

2.3. Proximate and amino acid analysis

Two selected representative hydrolysates (**CaFi55** and **CaFi63**) and a sample of raw *C. finmarchicus* biomass were analyzed for protein, fat and ash content, and amino acid composition. Total nitrogen of each sample was measured according to the Nordic Committee on Food Analysis method NMKL 6, based on the Kjeldahl method; the values were converted to percentage protein using the standard protein conversion factor of 6.25; fat and ash content were determined according to 2009/152/EU mod; amino acid composition was determined based on the method ISO 13903:2005 as stated by Commission Regulation EC 152/2009. The measurements of percentage protein from raw material and hydrolysates were used to calculate protein recovery.

2.4. Size exclusion chromatography

Analytical size exclusion chromatography (SEC) was performed as described previously (Wubshet et al., 2017). In short, lyophilized hydrolysates were rehydrated to a final concentration of 20 mg/mL. Injection solutions were prepared by filtration of each sample solution through a Millex-HV PVDF 0.45 μm 33 mm filter (MilliporeSigma, Burlington, MA, USA). Injection solutions of standards were prepared in mQ water at a concentration of 2 mg/mL. Chromatographic separation of standards and samples was performed with a Dionex UltiMate 3000 Standard System (Thermo Fisher Scientific, Waltham, MA, USA). An injection volume of 10 μL was used and separation was performed at room temperature using a BioSep-SEC-s2000 column, 300 × 7.8 mm i. d., 5 μm particle size (Phenomenex, Torrance, CA, USA). The mobile phase consisted of a mixture of acetonitrile and ultrapure water in a proportion of 30:70 (v/v), containing 0.05% trifluoroacetic acid. Isocratic elution was carried out using a flow rate of 0.9 mL/min for 20.0 min. Chromatographic runs were controlled from Chromeleon™ Chromatography Data System (CDS) software (Thermo Fisher Scientific, Waltham, MA, USA). From chromatographic runs of both the standards and hydrolysates, a UV trace of 214 nm was used. SEC chromatograms of the hydrolysates were converted to molecular weight distributions and weight average molecular weights (Mw) were calculated, as described previously (Wubshet et al., 2017), using a calibration curve constructed using molecular weight standards (Table S2, SI). These calculations were performed using PSS winGPC UniChrom V 8.00 (Polymer Standards Service, Mainz, Germany).

Fractionation was performed using the automated fraction collector in the same instrumental setup as the analytical runs. Mobile phase consisted of a mixture of acetonitrile and ultrapure water in a proportion of 30:70 (v/v), containing 0.2% formic acid. Isocratic elution was carried out using a flow rate of 0.9 mL/min for 20.0 min. Between 20.0 and 20.1 min the mobile phase was changed to NaH_2PO_4 (0.10 M) and maintained until 23.0 min for column cleaning. Elution conditions were restored between minute 23.0 and 23.1

Table 1
Overview of hydrolysis conditions for different batches.

Protease	30 min hydrolysis			60 min hydrolysis		
	0.1% w/w	0.5% w/w	1.0% w/w	0.1% w/w	0.5% w/w	1.0% w/w
Alcalase	CaFi01	CaFi02	CaFi03	CaFi04	CaFi05	CaFi06
Flavourzyme	CaFi07	CaFi08	CaFi09	CaFi10	CaFi11	CaFi12
Endocut 01	CaFi13	CaFi14	CaFi15	CaFi16	CaFi17	CaFi18
Bromelain	CaFi19	CaFi20	CaFi21	CaFi22	CaFi23	CaFi24
Flavorpro 786 MDP	CaFi25	CaFi26	CaFi27	CaFi28	CaFi29	CaFi30
Corolase 2 TS	CaFi31	CaFi32	CaFi33	CaFi34	CaFi35	CaFi36
Endocut 04L	CaFi37	CaFi38	CaFi39	CaFi40	CaFi41	CaFi42
Promod D24 MDP	CaFi43	CaFi44	CaFi45	CaFi46	CaFi47	CaFi48
Flavorpro 766 MDP	CaFi49	CaFi50	CaFi51	CaFi52	CaFi53	CaFi54
Flavorpro Umami 852 MDP	CaFi55	CaFi56	CaFi57	CaFi58	CaFi59	CaFi60
Endogenous proteases	CaFi61			CaFi62		
No protease	CaFi63					

and the column was equilibrated for an additional 42 min. Concentration of the sample for the semipreparative fractionation was 50 mg/mL and the injection volume was 35 μL. Four fractions were collected from 5 to 17 min. Retention times of the fraction were as follows: 5.00–10.25 min (**F1**), 10.25–11.25 min (**F2**), 11.25–12.00 min (**F3**) and 12.00–17.00 min (**F4**). Acetonitrile was removed using Savant SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA, USA). Samples were then frozen at −40 °C until lyophilized.

2.5. NMR measurements

All the samples were analyzed in duplicates. The NMR experiments were performed using a Bruker Ascend system (^{1}H operating frequency of 400 MHz) equipped with a sample changer and a gradient inverse triple-resonance 5 mm NMR probe optimized for ^{1}H and ^{13}C observation (Bruker Biospin). NMR solvent was prepared by mixing mQ H_2O: D_2O containing 0.05 wt % 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP): 200 mM phosphate buffer (pH 6.2) at a ratio of 17 : 2: 1. NMR samples of crude hydrolysates were prepared by dissolving the lyophilized hydrolysates in the NMR solvent at concentration of 15 mg/mL for the spectra acquisition. Samples of fractions separated by size-exclusion chromatography were prepared by dissolving the material in following concentrations: *c* (**F1**) = 12.5 mg/mL, *c* (**F2**) = 3.4 mg/mL, *c* (**F3**) = 6.3 mg/mL, *c* (**F4**) = 1.3 mg/mL. One-dimensional (1D) Nuclear Overhauser Effect Spectroscopy (NOESY) Proton NMR data was acquired using standard Bruker pulse sequence with 16 K data points, 6393.9 Hz spectral width and a total of 128 scans. Acquisition time and pulse relaxation delay were 2.5625 s and 5.00 s, respectively. FIDs were Fourier transformed, phase and baseline corrected before automatic alignment of signals in MestReNova, version 10.0.2 (Mestrelab Research S.L.). TSP was used as a chemical shift (δ) reference at 0.0 ppm.

2.6. ^{1}H NMR-based multivariate statistics

Peaks from the aligned NMR data were grouped in one matrix, normalized with SNV, and analyzed with PLS in MATLAB 2022b (The MathWorks, Inc, Natick, MA, USA). The peaks were identified with mspeaks and associated between samples if they were within 0.0025 ppm after alignment. The samples had between 500 and 1000 peaks, and the 500 peaks with the highest intensity that could be consistently grouped between samples were used in the data matrix. The replicates were treated as separate samples. The reference values consisted of one column for each enzyme, with 1 for the samples using that enzyme and 0 for all other samples, and one column each for the enzyme dose, hydrolysis duration, and enzyme dose multiplied by hydrolysis duration. Glycerol is present in varying quantities since it is added as a stabilizer to some of the enzymes. Since the signals from glycerol had a large impact on the PLS models, the glycerol signals were excluded, and the analysis was repeated without them.

2.7. DPP-IV inhibition assay

The activity of protein hydrolysates and selected fractions in inhibiting the enzyme dipeptidyl peptidase IV that degrades the GIP and GLP-1 peptides, involved in the regulation of blood glucose, was tested using a commercial fluorescence-based screening assay (DPP-IV Inhibitor Screening Assay Kit (ab133081) Abcam PLC, Cambridge, UK). Hydrolysates were tested at final assaying concentrations of 1.0 mg/mL and 0.1 mg/mL. SEC fractions (**F1** – **F4**) of the selected hydrolysate were tested at three concentrations: 1 mg/mL, 0.5 mg/mL and 0.1 mg/mL. Inhibitor samples were prepared by dissolving lyophilized hydrolysates in the assay buffer (20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, and 1 mM EDTA) to the corresponding stock solution and subsequently filtering them through a Millex-HV PVDF 0.45 μm 33 mm filter (MilliporeSigma, Burlington, MA, USA). The assay was performed according to the instructions from the manufacturer. In short, experiments were performed in 96-well microplates, with a final volume of 100 μL per well. Inhibitor-wells contained 10 μL of the test sample, 10 μL of the enzyme solution, 50 μL of the substrate solution and 30 μL of the assay buffer. To the initial activity-wells, assay buffer was added instead of inhibitor solution, and to the background-wells, assay buffer was added instead of both inhibitor and the enzyme solution. Sitagliptin (final assay conc. = 100 μM) was used as a positive control for the inhibition. The assay mixture was incubated for 30 min at 37 °C prior to acquiring emission values (λ_{exc} = 355 nm, λ_{em} = 455 nm). Fluorescence measurement was carried out using Synergy H1 hybrid multi-mode microplate reader (Synergy H1, Biotek, Winooski, VT, USA). Average value of background fluorescence was subtracted from all the readings of the samples. The % inhibition of each sample was calculated relative to the initial activity of the enzyme (without any inhibitor added) as follows:

$$\%\ Inhibition = \left(\frac{Initial\ enzyme\ activity - Enzyme\ activity\ with\ inhibitor}{Initial\ enzyme\ activity}\right) \times 100 \quad (1)$$

Crude hydrolysates were tested in triplicates. Determination of the IC_{50} value of the selected hydrolysates and fractions was performed by assaying the range of concentrations between 0.01 mg/mL to 5.00 mg/mL of inhibitors. Each concentration was tested in a multiplicate ($n = 3$ or $n = 6$). Results were processed in GraphPad Prism 9 (GraphPad Software Inc., Boston, MA, USA). The statistical significances of the measured bioactivities for the hydrolysates **CaFi01** – **CaFi60** were individually compared to the hydrolysates produced by endogenous hydrolysis (**CaFi61** and **CaFi62**), using Student's *t*-test in MATLAB 2023b.

2.8. ABTS radical scavenging assay

The antioxidant activity of hydrolysates was assessed using ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay. The assay was performed as described in the literature, with some modifications (Re et al., 1999). Briefly, $ABTS^{\bullet+}$ was prepared by reacting 7 mM aqueous ABTS solution with 2.45 mM aqueous ammonium persulfate. The mixture was allowed to stand in the dark, at the room temperature, for approximately 24 h, before diluting prepared $ABTS^{\bullet+}$ solution with the assay buffer (5 mM phosphate buffer, pH = 7.4) to the absorbance of 0.70 ± 0.05 at λ = 734 nm. Samples were prepared by dissolving freeze-dried

hydrolysates in mQ water to the stock concentrations of 2 mg/mL and subsequently filtering them through a Millex-HV PVDF 0.45 μm 33 mm filter (MilliporeSigma, Burlington, MA, USA). Experiments were conducted in triplicates, in 96-well microplates, with a final volume of 200 μL per well. Sample wells contained 10 μL of prepared sample solution to which 190 μL of diluted $ABTS^{\bullet+}$ solution was added. All hydrolysates were assayed at final concentration of 0.1 mg/mL. Trolox was prepared in a range of concentrations (40 μM–5 μM) and was used as antioxidant standard. L-ascorbic acid (assayed at final concentration of 5.0×10^{-3} mg/mL) was used as a positive control. Control absorbance wells contained 10 μL of mQ water to which 190 μL of diluted $ABTS^{\bullet+}$ solution was added. Background wells contained 10 μL of mQ water to which 190 μL of assay buffer was added. Microplate was incubated for 5 min in dark, at the room temperature and with shaking using microplate shaker (LP Vortex Mixer, Thermo Fisher Scientific, Waltham, MA, USA). After incubation, absorbance was measured at 734 nm (Synergy H1, Biotek, Winooski, VT, USA). Background absorbances were subtracted from all samples. The antioxidant activity was expressed as a percentage of decolorization of $ABTS^{\bullet+}$ solution, according to the formula:

$$\%\ Decolorization = \left(\frac{Control\ absorbance - Sample\ absorbance}{Control\ absorbance}\right) \times 100 \quad (2)$$

and as Trolox equivalent antioxidant capacity (TEAC), according to the formula:

$$Trolox\ eq\ (\mu M) = \left(\frac{Sample\ decolorization\ (\%) - b}{a}\right) \quad (3)$$

where a is the slope and b is the intercept obtained from linear regression from Trolox-caused decolorization as a function of Trolox concentration. The statistical significances of measured antioxidant activities for the hydrolysates **CaFi01** – **CaFi60** were individually compared to the hydrolysates produced by endogenous hydrolysis (**CaFi61** and **CaFi62**), using Student's *t*-test in MATLAB 2023b.

3. Results and discussion

A total of 63 hydrolysates (Table 1) were produced from *C. finmarchicus* using 10 commercial food-grade proteases (batches **CaFi01** – **CaFi60**) as well as endogenous proteolytic enzymes of the biomass (batches **CaFi61** and **CaFi62**) and one unhydrolyzed control (batch **CaFi63**). Strong proteolytic activities of endogenous enzymes have previously been reported for *C. finmarchicus* (Solgaard et al., 2007) and therefore it was studied here as a potential method for recovery of bioactive peptides. All prepared hydrolysates were characterized using size exclusion chromatography and nuclear magnetic resonance spectroscopy to study the effect of processing conditions (i,e., protease type, enzyme:substrate ratio and hydrolysis time) on composition of the final products. In addition, DPP-IV inhibition and antioxidant activity of the hydrolysates were studied.

3.1. Hydrolysis yields

Dissolved solids (measured as Brix, °Bx) and dry matter yield of all hydrolysis experiments are provided as Supplementary information (SI, Figs. S2 and S3, respectively). Different hydrolysis conditions provided a dry matter yield ranging between 5.1% and 8.4%, expressed as a percentage of mass of frozen raw material used in hydrolysis. The water content in the raw material was reported to be 78.8% (Vang et al., 2013). This value has been reported in the previous work, where the measurement of the water content was done on the *C. finmarchicus* biomass obtained through the standardized procedures by the same supplier (Calanus AS), so no significant differences were expected. Choice of the protease did not have significant effect on the yield (Fig. S3, SI). Nevertheless, some of the higher yielding proteases included Corolase 2 TS and Bromelain, both endo-protease preparations, followed by Flavorpro Umami 852 MDP and Endocut 04 L. The lowest yielding hydrolysis conditions included 1.0% Alcalase (w/w) used for 30 min, while the highest yield was achieved using Endocut 04 L, 1.0% w/w and 60 min hydrolysis. Since the choice of protease and hydrolysis time did not show

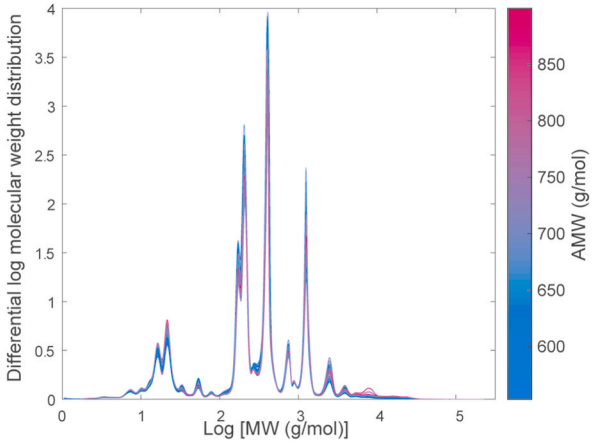

Fig. 1. Overlayed molecular weight distributions of 62 *C. finmarchicus* hydrolysates (**CaFi01** – **CaFi62**) produced using varying processing conditions.

systematic influence on the yield, the observed variations are potentially the result of variation in the dry matter content in the raw material. It is interesting to note that the protein yields of batches **CaFi61** and **CaFi62** (hydrolysis by endogenous enzymes) were comparable to the yields of the rest of the conditions. This information highlights the presence of effective endogenous proteases in *C. finmarchicus*, and the strong influence they exert on the process.

3.2. Size-exclusion chromatography and molecular weight distribution

Molecular weight distributions (MWD) of *C. finmarchicus* hydrolysates produced using different conditions were studied (Fig. 1). It is known from the literature that crustacean bodies contain a multitude of short peptides and single amino acids (Cowey and Corner, 1963; Lazcano-Perez et al., 2012). In agreement with this, MWD of our hydrolysates revealed an abundance of low molecular weight peptides. Different hydrolysis conditions (e.g., % enzyme and hydrolysis time) showed minor impact on the SEC profiles of hydrolysates and corresponding MWD, compared to hydrolysates from other biomasses, like poultry (Lapeña et al., 2018; Lindberg et al., 2021b).

Average molecular weight (AMW) values of *C. finmarchicus* hydrolysates produced using commercial proteases were ranging from 553.30 g/mol (**CaFi05**, 0.5% w/w Alcalase, 60 min) to 850.67 g/mol (**CaFi13**, 0.1% w/w, Endocut 01, 30 min, Figs. S4 and SI). Interestingly, hydrolysates from the batches with endogenous enzymes (i.e., **CaFi61** and **CaFi62**) showed AMW (899.65 g/mol and 754.29 g/mol, respectively) and MWD profiles comparable to the hydrolysates produced with commercial proteases (**CaFi01** to **CaFi60**). It is well known from the literature that *C. finmarchicus* contains proteases that can rapidly digest the animal's protein after its death (Bergvik et al., 2012; Solgaard et al., 2007). These crustacean enzymes were characterized as metallo, serine and aspartic proteases using specific inhibitors (Solgaard et al., 2007). On the other hand, reports about protein hydrolysates derived from *C. finmarchicus* are scarce and limited mainly to feed applications or mentioned in context of extraction of lipids. It is therefore not known how the commercial protease preparations cooperate or compete with endogenous proteases from *C. finmarchicus*. This study shows that the activity of endogenous enzymes in this case is very strong and likely has a decisive impact on the quality of the final product.

3.3. ^{1}H NMR of the hydrolysates

In order to further investigate if processing conditions have a particular effect on the type of peptides generated, ^{1}H NMR of all the hydrolysates were studied. Chemical shifts of the representative ^{1}H NMR spectrum of *C. finmarchicus* hydrolysate were tentatively assigned (Fig. 2). The ^{1}H NMR spectra revealed that, in addition to diagnostic peaks attributed to amino acid chains, the hydrolysates constituted abundant signals assigned to metabolites such as dimethyl glycine (DMG) and trimethyl amine oxide (TMAO). Similar to the MWD, no visual differences were observed in the NMR fingerprints of the 63 hydrolysates. However, classification based on ^{1}H NMR fingerprints (i.e., PLS analysis) revealed a slight grouping according to the enzymes used (Fig. 3). A closer look at the loadings indicated that the classification was driven by the signals from constituents of the commercial proteases used (e.g., the stabilizing agent glycerol) instead of the *C. finmarchicus* peptides. However, even after removing such signals, subtle but clear structures of classification could be observed for selected classes of enzymes. This is visible in the PLS X-scores plot (Fig. S5, SI) where data from a pair of enzymes (Flavourpro Umami 852 MDP and Alcalase) were used as an example. To verify the PLS models, new PLS models were created using samples from one or two enzymes, with similar results. While the differences in NMR profiles are mainly a result of parameters such as enzyme dose, hydrolysates produced using the different enzymes were shown to result in different ^{1}H NMR spectra. This indicates that, despite the strong endogenous activity discussed in sections 3.1. and 3.2., addition of commercial enzymes may result in altered hydrolysate composition. It is important to note, however, that these differences are subtle and their significance for the quality of the product could be minuscule.

3.4. DPP-IV inhibiting and antioxidant activity

Inhibitory activity of crude hydrolysates on the DPP-IV enzyme was measured at two concentrations: 0.1 mg/mL and 1 mg/mL. Lower concentrations did not cause inhibition of the enzyme (data not shown), while at 1 mg/mL all the crude hydrolysates (**CaFi01** – **CaFi62**) resulted in significant inhibition (36.7–71.5%, Fig. 4, Table S4, SI). Previous studies on hydrolysates from another crustacean species, Antarctic krill, reported a similar range of inhibition (Ji et al., 2017; Naik et al., 2021). In accordance with limited variation in chemical composition of *C. finmarchicus* hydrolysates, the measured DPP-IV inhibitions did not show large variations. To weigh the

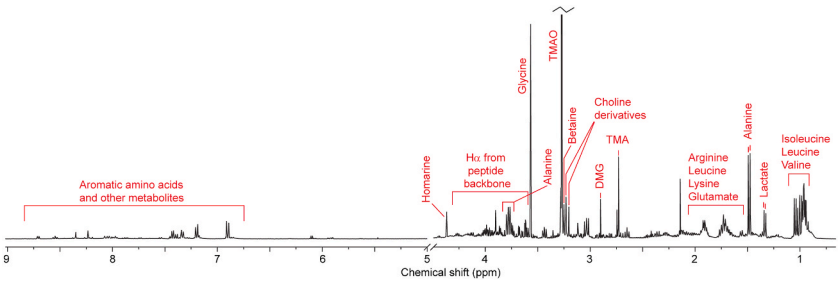

Fig. 2. Representative ^{1}H NMR spectrum of crude *C. finmarchicus* hydrolysate (**CaFi55**). Assignment of diagnostic peaks for peptides, amino acids and other metabolites was made according to published chemical shift values for related sample materials (Hansen et al., 2013; Sundekilde et al., 2018).

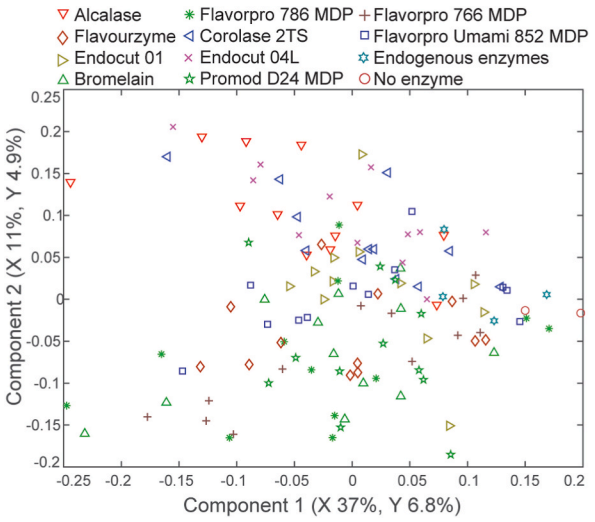

Fig. 3. A PLS X-scores plot revealing the enzyme grouping. Alcalase, Corolase, Endocut 01 and 04 L stand out. When signals from glycerol are included, they dominate the loadings separating these enzymes from the others. In the figure, the signals from glycerol have been excluded, but the enzyme grouping remains.

effect of different hydrolysis conditions, DPP-IV inhibitions of hydrolysates **CaFi01** – **CaFi60** were compared to the bioactivities of endogenous batches (**CaFi61** and **CaFi62**), by Student's *t*-test (Fig. S6, SI). Hydrolysates produced by Alcalase, Flavourzyme and Endocut 01 in general showed lower bioactivities compared to **CaFi61** and **CaFi62**, which was statistically significant ($p < 0.05$). Flavorpro Umami 852 MDP yielded one hydrolysate (**CaFi55**) with higher DPP-IV inhibition, that had statistical significance. However, for most of the hydrolysates, these differences are not practically significant given that this is a single-concentration screening test, where variations between replicates have to be taken into consideration. Nevertheless, a strong contrast was observed in the activity of the unhydrolyzed control (**CaFi63**, 10.6 ± 2.0% inhibition) compared to the products of hydrolyses.

Antioxidative properties of *C. finmarchicus* hydrolysates were evaluated by measuring Trolox Equivalent Antioxidant Capacity (TEAC). The TEAC values for the hydrolysates were measured to be between 19.54 and 25.42 μM for 0.1 mg/mL (Fig. 4). Similar to the DPP-IV, the lowest activity was observed for the unhydrolyzed control **CaFi63**. The results were also analyzed by Student's *t*-test (Fig. S7, SI). Certain samples showed statistically significant differences ($p < 0.05$), which were more systematic compared to the DPP-IV inhibiting activity. For example, bromelain and Corolase 2 TS showed slightly positive influence on the antioxidant potential, as well as hydrolysis time in general. However, those differences were again not practically significant, considering that the most active sample had only about 1.3-fold higher activity compared to the unhydrolyzed control.

In general, no clear trend was observed between different hydrolysis conditions and measured bioactivities. The main reason for this is likely a high degree of hydrolysis by endogenous proteases of *C. finmarchicus*, which is evident from the SEC analyses of **CaFi61** and **CaFi62**. High degree of hydrolysis ensures good product yield, and typically correlates with the bioactivity. However, the specificity of the protease plays a more influential role in producing peptide sequences which exert desired properties. In all *C. finmarchicus* hydrolyses carried out in this work, the specificity of added proteases was hindered by a strong activity of endogenous enzymes. Although some samples showed statistically significant differences in bioactivities compared to endogenous batches, these

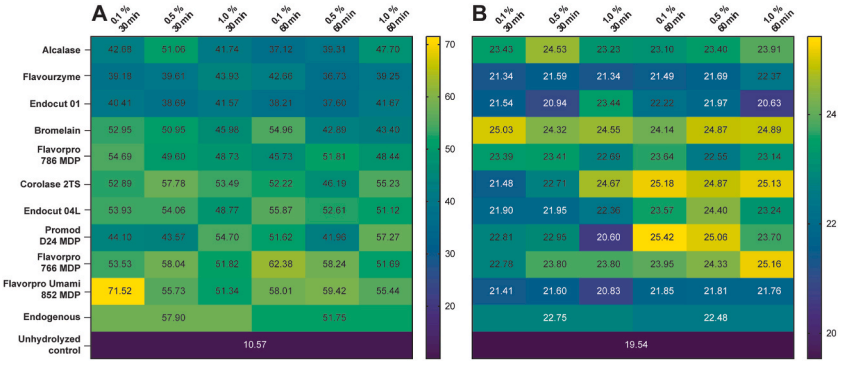

Fig. 4. Overview of bioactive properties of *C. finmarchicus* hydrolysates: A) DPP-IV inhibition activity of **CaFi01** – **CaFi63** at 1 mg/mL; B) Antioxidant activity of **CaFi01** – **CaFi63** at 0.1 mg/mL, expressed as TEAC values. Color bars represent the magnitude of % inhibition. For clarity of presentation, standard deviations are presented as Supplementary information (Tables S4, S5 and S6, SI). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

results should be interpreted within context of practical significance.

3.5. Isolation and characterization of potent peptide fraction

To elucidate the capacity of peptides to inhibit the enzyme dipeptidyl peptidase IV, one protein hydrolysate from *C. finmarchicus* was selected for further analyses. Since the chemical fingerprints (MWD and ^{1}H NMR) as well as DPP-IV inhibiting activity of the hydrolysates were comparable, batch **CaFi55**, was selected as a representative sample for further investigation. This hydrolysate was produced at favorably shorter hydrolysis time (30 min) and lowest enzyme loading (0.1%), using Flavorpro Umami 852 MDP. Proximate composition as well as amino acid composition of **CaFi55** is presented in the Supplementary information (Fig. S8 and Table S7). The IC_{50} value of **CaFi55** for the inhibition of DPP-IV was determined to be 0.93 ± 0.11 mg/mL. It is important to consider that the significant amount of inorganic material (26.9 g ash/100 g dry matter, Fig. S8, SI) measured as % ash in the proximate analysis could lead to underestimation of bioactivity (i.e., the presented IC_{50} value). The crude hydrolysate **CaFi55** was further fractionated using SEC, to provide four fractions: **F1**, **F2**, **F3** and **F4** at an approximate yield ratio of 7:4:2:1, respectively (Table 2). The average MW values of fractions were 1783 g/mol (**F1**), 557 g/mol (**F2**), 250 g/mol (**F3**) and 131 g/mol (**F4**).

The four molecular weight fractions were tested for DPP-IV inhibition (Fig. 5 and Fig. S9, SI). Fraction **F2** showed the best inhibition among the four fractions (72.2 ± 2.5% at 1 mg/mL). Based on the calculated AMW, this fraction is composed of small peptides with an average chain length of five amino acids (a single amino acid is approximated to 110 g/mol). This is consistent with previous findings which attributed DPP-IV inhibition of hydrolysates to small peptides of similar length (Power et al., 2014). Interestingly, fraction **F4** also showed relatively high inhibition (63.0 ± 2.1% at 1 mg/mL). However, ^{1}H NMR analysis of **F4** (Fig. S10, SI) showed abundant peaks attributed to low molecular weight metabolites and relatively low signals from free amino acids and peptide side chains. Therefore, this fraction was not considered for further analyses. IC_{50} values were determined for the two major fractions **F2** and **F1**, and they were measured to be 0.70 ± 0.07 mg/mL and 1.98 ± 0.17 mg/mL, respectively (Fig. 6). In contrast to **F2**, **F1** comprised of relatively large peptides, with an average chain length of sixteen amino acids.

The DPP-IV inhibitory potential of a *C. finmarchicus* hydrolysate, reported here for the first time, was better than those previously reported for marine hydrolysates from Antarctic krill (Ji et al., 2017), European pilchard (Rivero-Pino et al., 2020), salmon skin collagen (Jin et al., 2020), or boarfish hydrolysate (Harnedy-Rothwell et al., 2020), and are comparable to other potent inhibitors reported (Neves et al., 2017; Zhang et al., 2022).

The merit of enzymatic protein hydrolysis is the tunability of the process to tailor-make a peptide product of specific quality. In the presented work, we have studied the effect of the tunable parameters (i.e., enzyme type, enzyme dose and hydrolysis time) on bioactivity (i.e., DPP-IV inhibition and antioxidant activity) of the resulting peptides. A previous study has shown that processing conditions used in enzymatic hydrolysis of poultry byproduct hydrolysates have significant effect on bioactivity of the resulting peptides (Sorokina et al., 2022). However, in the current study, the impact of the different processing conditions was very limited on bioactivity, molecular weight distribution and protein yield of *C. finmarchicus* hydrolysates. This observation was, as discussed above, attributed to the strong effect of endogenous enzymes present in the raw material used (*C. finmarchicus*). Therefore, if recovery of bioactive peptides is the main aim, hydrolysis by endogenous enzymes should be considered as a plausible approach for *C. finmarchicus*. A similar approach utilizing autolytic process for recovery of peptides has already been demonstrated for Antarctic krill (Wang et al., 2015) and Pacific whiting (Mazorra-Manzano et al., 2012).

4. Conclusion

C. finmarchicus is a valuable and underutilized marine resource. With possible challenges in maintaining safe food supply in the future, it could be an important source of nutrients and nutraceuticals. In this work, we have described a set of *C. finmarchicus* hydrolysates, produced by using different conditions of enzymatic hydrolysis. Impact of hydrolysis conditions on the protein yield, molecular weight distribution and bioactivity of resulting hydrolysates was studied. In general, process conditions had limited impact on the measured quality characteristics of the final product. Moreover, hydrolysis with endogenous enzymes resulted in MWD and bioactivity comparable to hydrolysates obtained by commercial enzymes. Crude hydrolysates showed promising DPP-IV inhibitory activity as well as antioxidant activity. Refining the crude hydrolysate by SEC fractionation resulted in potent peptide fractions, containing mostly oligopeptides, which was confirmed by SEC and ^{1}H NMR analyses. The IC_{50} value of the active fraction was 0.70 ± 0.07 mg/mL, which was significantly more potent than the original crude hydrolysate (IC_{50} = 0.93 ± 0.11 mg/mL).

Declaration of competing interest

Authors have no conflicts of interests to declare.

Table 2
Fractions of **CaFi55** isolated based on size-exclusion chromatography.

Fractions	CaFi55	F1	F2	F3	F4
Ret. Time/min	/	05.00–10.25	10.25–11.25	11.25–12.00	12.00–17.00
Yield	/	43.1%	26.2%	13.6%	6.5%
AMW/g/mol	666	1783	557	250	131

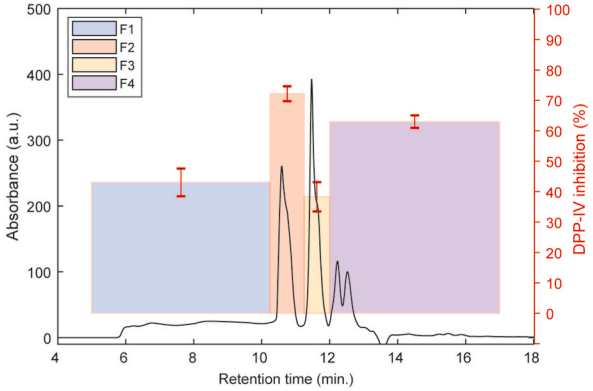

Fig. 5. Size exclusion chromatogram from the semipreparative fractionation of crude *C. finmarchicus* hydrolysate (**CaFi55**) with overlayed bar plot of %DPP-IV inhibition measured at 1.0 mg/mL for collected fractions **F1**, **F2**, **F3** and **F4**. The width of each bar represents fraction collection period for individual fractions.

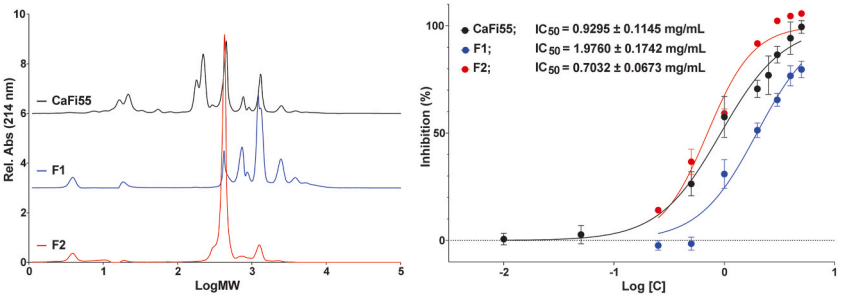

Fig. 6. Left: Size exclusion chromatogram of **CaFi55** overlayed with fractions **F1** and **F2** (c = 10 mg/mL, V (injection) = 10 μL); right: dose-response curves and the average values of single concentration measurements of DPP-IV inhibition for **CaFi55** and fractions **F1** and **F2**; inserted in the top-left corner are the IC_{50} values of DPP-IV inhibition for CaFi55 and fractions **F1** and **F2**.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2023.102820.

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