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1 **Proteomic characterization of pilot scale hot-water extracts from the industrial carrageenan red**
2 **seaweed *Eucheuma denticulatum***

3

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12

13 **Abstract:**

14 Seaweeds have a long history as a resource for polysaccharides/hydrocolloids extraction for use in the food
15 industry due to their functionality as stabilizing agents. In addition to the carbohydrate content, seaweeds
16 also contains a significant amount of protein, which may find application in food and feed. Here, we
17 present a novel combination of transcriptomics, proteomics, and bioinformatics to determine the protein
18 composition in two pilot-scale extracts from *Eucheuma denticulatum* (Spinosum) obtained via hot-water
19 extraction. The extracts were characterized by qualitative and quantitative proteomics using LC-MS/MS and
20 a *de-novo* transcriptome assembly for construction of a novel proteome. Using label-free, relative
21 quantification, we were able to identify the most abundant proteins in the extracts and determined that
22 the majority of quantified protein in the extracts (>75%) is constituted by merely three previously

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23 uncharacterized proteins. Putative subcellular localization for the quantified proteins was determined by
24 bioinformatic prediction, and by correlating with the expected copy number from the transcriptome
25 analysis, we determined that the extracts were highly enriched in extracellular proteins. This implies that
26 the method predominantly extracts extracellular proteins, and thus appear ineffective for cellular
27 disruption and subsequent release of intracellular proteins. Ultimately, this study highlight the power of
28 quantitative proteomics as a novel tool for characterization of alternative protein sources intended for use
29 in foods. Additionally, the study showcases the potential of proteomics for evaluation of protein extraction
30 methods and as powerful tool in the development of an efficient extraction process.

31

32 **Keywords**

33 *Eucheuma denticulatum*; hot-water protein extraction; quantitative proteomics; *de novo* quantitative
34 transcriptomics; bioinformatics; subcellular localization

35

36 **1. Introduction:**

37 Seaweeds are known to contain numerous compounds of interest, such as polysaccharides, proteins and
38 other compounds with health beneficial properties such as anti-inflammatory, anti-oxidant, and anti-cancer
39 (Holdt & Kraan, 2011; Leandro et al., 2020). The industry to produce hydrocolloids from seaweed is well
40 established, and the hydrocolloids are used as e.g. stabilizing agents in toothpaste, canned whipped cream,
41 and as meat glue. The production of red carrageenan accounts for 54,000 ton/year and constitutes the
42 majority of the total hydrocolloids sold worldwide (also incl. alginate and agar). Carrageenan is extracted
43 from 212,000 ton dried seaweed, and brings in a value of 530 million USD (Porse, 2018). *Eucheuma*
44 *denticulatum* is among the most cultivated and harvested red seaweed species for the carrageenan
45 industry. However, at present carrageenan is extracted in a process, which extracts carrageenan as the only
46 compound whereas proteins and other compounds are not extracted. The most common industrial method

47 to extract carrageenan from *Eucheuma denticulatum* uses hot water at high pH. If further extraction of
48 other compounds such as proteins could be made prior to or as part of the industrial hot water extraction
49 without compromising the existing carrageenan extraction, this could be of interest, since the amount of
50 biomass available is large. Proteins from *E. denticulatum* were shown to constitute only 3.8% of dry
51 biomass, but were of high quality with respect to their amino acid profile (Naseri, Jacobsen, et al., 2020).
52 Moreover, the obtained proteins are comparable to beef in regard to the branched chained amino acids
53 (i.e. leucine, isoleucine, and valine) that are of interest due to their muscle building properties.

54 In addition to the general health benefits from ingestion (Gomez-Zavaglia, Prieto Lage, Jimenez-Lopez,
55 Mejuto, & Simal-Gandara, 2019; Peñalver et al., 2020), seaweed may also be a source of bioactive peptides
56 that could exhibit a direct biological purpose or be utilized as functional food ingredients. These peptides
57 can be released through bio-processing of proteins extracts using e.g. enzymatic hydrolysis or fermentation
58 (Admassu, Gasmalla, Yang, & Zhao, 2018). In the past decade, peptides derived from seaweed proteins with
59 e.g. renin-inhibitory (Fitzgerald et al., 2012), ACE-inhibitory (Furuta, Miyabe, Yasui, Kinoshita, & Kishimura,
60 2016), antioxidant (Cian, Garzón, Ancona, Guerrero, & Drago, 2015), and antidiabetic (Harnedy &
61 FitzGerald, 2013b) activities have been identified. Common for all bioactive peptides is that they were
62 identified in enzymatic hydrolysates by a non-targeted trial-and-error approach. This methodology,
63 commonly employed in the food industry, requires numerous costly and time-demanding steps of
64 hydrolysis, separation, isolation, identification, and finally *in vitro* or *in vivo* verification of activity. In
65 contrast, an orthogonal approach utilizing bioinformatic prediction of bioactive peptides, is gathering
66 increased attention (Tu, Cheng, Lu, & Du, 2018). This method reduces cost and work load tremendously,
67 and allows for targeted peptide release by enzymatic hydrolysis. With recent advances in bioinformatic
68 prediction of peptide functionality (García-Moreno, Jacobsen, et al., 2020; Mooney, Haslam, Holton,
69 Pollastri, & Shields, 2013; Mooney, Haslam, Pollastri, & Shields, 2012; Olsen et al., 2020; Panyayai et al.,
70 2019), and the growing availability of peptide databases (Chen et al., 2013; Liu, Baggerman, Schoofs, &
71 Wets, 2008; Minkiewicz, Iwaniak, & Darewicz, 2019; G. Wang, Li, & Wang, 2009), the primary prerequisite

72 for the analysis is the availability of protein sequences and quantitative information on protein
73 composition. Recently, we employed quantitative proteomics for identification of abundant proteins
74 followed by bioinformatic prediction (EmulsiPred source code freely available at
75 <https://github.com/MarcatiliLab/EmulsiPred>) to identify a number of highly functional emulsifier peptides
76 from potato (García-Moreno, Gregersen, et al., 2020) as well predicting probable emulsifier and antioxidant
77 peptides in hydrolysates from fish processing side streams following LC-MS/MS analysis (Jafarpour, Gomes,
78 et al., 2020; Jafarpour, Gregersen, et al., 2020). Nevertheless, proteomic quantification of the starting
79 material is an absolute necessity in order to maximize the yield of peptide release. Here, we present a
80 proteomic characterization of two industrially relevant, pilot-scale extracts from *E. denticulatum* obtained
81 by hot-water extraction. Protein identification is based on a *de novo* transcriptome assembly for creating a
82 novel reference proteome. Furthermore, we present a novel approach for quantifying proteins based on
83 non-tryptic peptides, and correlate protein abundance with quantitative transcriptomics. Using
84 bioinformatic prediction of protein subcellular origin, we are able to determine enrichment of certain
85 protein classes in the extracts.

86

87 **2. Materials and Methods**

88 *2.1. Materials*

89 Two *Eucheuma denticulatum* protein extracts obtained using near-neutral, hot-water extraction were
90 supplied by the global food ingredient provider CP Kelco. Protein extract A was obtained by dispersing the
91 raw seaweed in deionized water (pH adjusted to 8.9 with sodium carbonate) and applying continuous
92 stirring at 95°C for 5 h. The slurry was subsequently filtered in a Büchner funnel followed by diafiltration
93 using a 300 kDa MWCO membrane. The retentate was washed with three volumes of 0.9% sodium chloride
94 in deionized water, and all permeates were subsequently pooled. The pooled permeate was then
95 concentrated using a 1 kDa MWCO membrane, and the retentate lyophilized to yield the final protein

96 extract A. Protein extract B was obtained similarly to extract A, but with stirring at 90°C for 16 h before
97 filtering, diafiltration, concentration, and lyophilization. Furthermore, the lyophilized retentate was
98 dissolved in deionized water, the pH was adjusted to 2.9 with nitric acid, and the mixture was stirred at
99 room temperature for 1 h. Precipitated protein was isolated by centrifugation and washed twice with
100 isopropanol before air drying and lyophilization to yield the final protein extract B. The total protein
101 content of protein extracts A and B (by Kjeldahl-N) was 7.1% and 70% (w/w), respectively, using a nitrogen-
102 to-protein conversion factor of 6.25 (CP Kelco supplied information). All chemicals used were of analytical
103 grade.

104

105 *2.2. Total soluble protein*

106 Protein extracts A and B were solubilized to an estimated protein concentration of 2 mg/mL in ddH₂O and
107 in 200 mM NH₄HCO₃ with 0.2% SDS for maximal solubilization compatible with the Qubit protein assay.
108 Following solvent addition, samples were vortexed for 30 s, sonicated for 30 min, and left overnight on a
109 Stuart SRT6 roller mixer (Cole-Parmer, UK). The next day, samples were sonicated for 30 min, left on a roller
110 mixer for 60 min, and centrifuged at 3,095 RCF (ambient temperature) for 10 min in a 5810 R centrifuge
111 (Eppendorf, Germany), prior to aliquoting the supernatant. The total soluble protein content of the samples
112 in both solvents, was quantified using Qubit protein assay (Thermo Scientific, Germany) according to the
113 manufacturer guidelines.

114

115 *2.3. 1D-SDS-PAGE and in-gel digestion.*

116 Protein extracts A and B were solubilized with 2% SDS in 200 mM ammonium bicarbonate (pH 9.5) to a final
117 protein/peptide concentration of 2 mg/mL based on protein content by Kjeldahl-N. Alkaline buffer with
118 detergent was used to maximize protein solubilization. Solubilization was further promoted by. Samples
119 were vortexed for 2 min, sonicated for 30 min, and subsequently centrifuged at 3,095 RCF for 15 min to

120 precipitate solids. SDS-PAGE analysis was performed on precast 4-20% gradient gels (GenScript, USA) in a
121 Tris-MOPS buffered system under reducing conditions according to manufacturer guidelines. Briefly, 20 µg
122 protein/peptide was mixed with reducing (final DTT concentration 50 mM) SDS-PAGE sample buffer and
123 subsequently denatured at 95 °C for 5 min prior to loading on the gel. As molecular weight marker, PIERCE
124 Unstained Protein MW Marker P/N 26610 (ThermoFisher Scientific, USA) was used. Protein visualization
125 was achieved by using Coomassie Brilliant Blue G250 staining (Sigma-Aldrich, Germany) and imaging with a
126 ChemDoc MP Imaging System (Bio-Rad, USA).

127 Proteins were in-gel digested according to Shevchenko et al. (Shevchenko, Wilm, Vorm, & Mann, 1982) and
128 Fernandez-Patron et al. (Fernandez-Patron et al., 1995), as previously described (García-Moreno,
129 Gregersen, et al., 2020). Briefly, each gel lane from the gradient gel was excised with a scalpel and divided
130 into 6 fractions guided by the MW marker (<14kDa; 14-25kDa; 25-45kDa; 45-66kDa; 66-116kDa; >116kDa).
131 Individual fractions were cut into 1x1 mm pieces before being subjected to washing, reduction with DTT,
132 Cys alkylation with iodoacetamide, and digestion with sequencing grade modified trypsin (Promega,
133 Madison, WI, USA). Following digestion, peptides were extracted, dried down by SpeedVac, and suspended
134 in 0.1% (v/v) formic acid (FA), 2% acetonitrile (ACN) (v/v). Next, peptides were desalted using StageTips
135 (Fernandez-Patron et al., 1995; Rappsilber, Mann, & Ishihama, 2007), dried down by SpeedVac, and finally
136 suspended in 0.1% (v/v) FA, 2% ACN (v/v) for LC-MS/MS analysis.

137

138 *2.4. De novo transcriptome assembly.*

139 The transcriptome of *E. denticulatum* was downloaded from the NCBI SRA database
140 (<https://www.ncbi.nlm.nih.gov/sra/SRX2653634>). The raw reads were preprocessed by Trimmomatic
141 software to filter short sequences (less than 36 bp) and to trim low-quality ends (Bolger, Lohse, & Usadel,
142 2014). Processed reads were then assembled *de novo* into contigs using Trinity with default parameters
143 (Grabherr et al., 2011). Overall, 9458 contigs were assembled with an average length of 1021 bp.

144

145 *2.5. Transcript annotation, abundance estimation and protein database construction.*

146 The potential protein-coding sequences were predicted by TransDecoder based on the length of open
147 reading frames and nucleotide composition (Grabherr et al., 2011). Candidate sequences were annotated
148 by BlastP and BlastX search against SwissProt database (Madden, 2013) with the cutoff E-value of 1E-5 as
149 well as by HMMER (Finn, Clements, & Eddy, 2011) search against Pfam database (El-Gebali et al., 2018; Finn
150 et al., 2010). An alignment E-value of 1E-5 means that a homology hit has a 1 in 100,000 probability of
151 occurring by chance alone, therefore we chose this threshold to get only high-quality homologous proteins
152 hits.

153 The abundance of the transcripts (transcripts per megabase, TPM) was calculated by re-aligning reads to
154 the assembled contigs using RSEM (RNA-Seq by Expectation-Maximization) estimation method included in
155 Trinity software (Grabherr et al., 2011). Obtained transcript abundance matrix was joined with Blastp-
156 annotated transcripts to attain a list of highly expressed proteins.

157

158 *2.6. Prediction of subcellular localization using deepLoc*

159 All proteins in the final database were analyzed by deepLoc (Almagro Armenteros, Søndery, Søndery,
160 Nielsen, & Winther, 2017) using the freely available web-tool
161 (<http://www.cbs.dtu.dk/services/DeepLoc/index.php>). All searches were performed using the BLOSUM62
162 protein encoding to achieve a probability based subcellular localization for use in enrichment analysis on
163 both transcriptome and protein level.

164

165 *2.7. LC-MS/MS analysis*

166 Tryptic peptides were analyzed by an automated LC–ESI–MS/MS consisting of an EASY-nLC system (Thermo
167 Scientific, Bremen, Germany) on-line coupled to a Q Exactive mass spectrometer (Thermo Scientific) via a
168 Nanospray Flex ion source (Thermo Scientific), as previously reported (García-Moreno, Gregersen, et al.,
169 2020). Separation of peptides was achieved by use of an Acclaim Pepmap RSLC analytical column (C18, 100
170 Å, 75 µm. × 50 cm (Thermo Scientific)). Instrumental settings, solvents, flows, gradient, and acquisition
171 method were identical to what was described previously.

172

173 *2.8. Proteomics data analysis*

174 Protein identification and quantification was performed using MaxQuant 1.6.0.16. (Cox & Mann, 2008;
175 Tyanova, Temu, Sinitcyn, et al., 2016) using the *de-novo* proteome assembled from the transcriptomic
176 analysis. Initially, standard settings were employed using specific digestion (Trypsin/P, 2 missed cleavages
177 allowed, minimum length 7 AAs) and false discovery rate (FDR) of 1% on both peptide and protein level.
178 FDR was controlled using reverse decoy sequences and common contaminants were included. Protein
179 quantification was obtained with including both unique and razor peptides. Samples were analyzed as six
180 fractions with boosted identification rates by matching between runs and dependent peptides enabled. The
181 iBAQ algorithm (Schwanhüusser et al., 2011) was used for relative in-sample protein quantification. iBAQ
182 intensities were normalized to the sum of all iBAQ intensities after removal of reverse hits and
183 contaminants, to obtain the relative iBAQ (riBAQ), as previously described (García-Moreno, Gregersen, et
184 al., 2020; Shin et al., 2013).

185 MS-data were furthermore analyzed both semi-specifically (tryptic *N*- or *C*-terminus) and unspecifically (no
186 terminal restrictions) in MaxQuant. All settings were maintained except for applying unspecific digestion
187 with peptide length restrictions from 4 to 65 AAs. Additional unspecific searches with peptide and protein
188 level FRD of 5% and 10% as well as semi-specific searches with peptide and protein level FRD of 5% was

189 conducted to increase identification rates and sequence coverage for comparison and data quality
190 assessment.

191 Relative quantification with iBAQ employs strict tryptic restrictions to peptide termini and consequently,
192 this type of quantifications is not possible for semi-specific and unspecific searches. In order to compare
193 and evaluate the semi-specific and unspecific results, we introduced two additional quasi-quantitative
194 relative metrics: i) relative intensity, I_{rel} and ii) length-normalized relative intensity, I_{rel}^L . They were defined
195 as:

$$196 \quad I_{rel}(n) = \frac{I_n}{\sum_{n=1}^p I_n} * 100\% \quad (\text{Eq. 1})$$

$$197 \quad I_{rel}^L(n) = \frac{I_n/L_n}{\sum_{n=1}^p I_n/L_n} * 100\% \quad (\text{Eq. 2})$$

198 Where I_n is the intensity of protein n of p quantified proteins in a given sample and L_n is the length of
199 protein n , based on the processed protein database. For evaluation of the two metrics, relative protein
200 abundance was plotted as scatter plots between the different analysis conditions and the Pearson
201 correlation coefficient (PCC) was calculated in Perseus (Tyanova, Temu, Sinitcyn, et al., 2016).

202 For final protein quantification, MS data were analyzed as both tryptic and semi-tryptic digests using the
203 following optimized search criteria: Peptides per protein ≥ 2 (razor and unique), protein FDR = 0.05,
204 unmodified peptide score > 40 , peptide FDR = 0.005. Match between runs and dependent peptides were
205 both disabled. This was done to alleviate false positive identifications and increase quantitative validity.
206 Increasing FDR to 5% for the tryptic analysis did not affect identification and quantification due to the
207 applied score threshold.

208

209 *2.9. Comparative analysis of transcriptomic and proteomic data*

210 Comparative analysis was done on both the protein and subcellular levels. To estimate molar transcript
211 abundance, we calculated the relative TPM (rTPM) for the individual proteins to the sum of TPMs for all
212 1628 proteins in the database. Using the predicted subcellular localization, we then estimated the relative
213 distribution of proteins based on the transcriptome using rTPM. Finally, we correlated the transcriptome-
214 based protein distribution with the actual protein distribution for the extracts in a relative, quantitative
215 manner.

216

217 *2.10. Data analysis and visualization*

218 Statistical and correlation analysis of transcriptome and MS data was performed in Perseus 1.6.1.3
219 (Tyanova & Cox, 2018; Tyanova, Temu, Sinitcyn, et al., 2016). Venn diagrams were plotted with jvenn
220 (Bardou, Mariette, Escudié, Djemiel, & Klopp, 2014). Additional data visualization was obtained using
221 OriginPro 8.5.0 SR1 (OriginLab Corporation, Northampton, MA, USA) and figures assembled in their final
222 form using INKSCAPE version 0.92.3 (<https://inkscape.org/>).

223

224 **3. Results and Discussion:**

225 *3.1. Transcriptome assembly, protein annotation, and subcellular localization*

226 The transcriptome of *Eucheuma denticulatum* was de novo assembled using publicly deposited
227 transcriptome data at NCBI SRA database. The quality of the assembly was estimated by basic contig
228 statistics and percentage of the remapped reads. Both metrics indicated a high quality of the assembly with
229 an N50 value of 1891bp (Table A.1) and more than 90% of the reads mapped back to the contigs (Table
230 A.2). Based on the transcriptomic information, an *E. denticulatum* protein database was constructed for
231 subsequent mass-spectrometry (MS) analysis. First, the protein-coding sequences were predicted and

232 identified their by BlastX and BlastP search as well as their protein family by searching against Pfam
233 database. Then the transcript expression level was calculated in terms of transcripts per kilo megabase
234 (TPM) and removed proteins with TPM below 100, which resulted in 1628 proteins retained for the
235 database. The TPM threshold was applied in order to filter out any potentially erroneous reads. Although
236 this may in fact also filter some proteins with low copy numbers from the database, the primary objective
237 was to identify highly expressed and extracted proteins, and consequently do not regard this to have
238 substantial influence. A full list of protein accessions and their associated TPMs, rTPMs, Pfam functions,
239 BlastX targets, and BlastP targets can be found in Table A.3 and in the linked Mendeley data repository. The
240 *de-novo* protein database for *E. denticulatum* can be found in .fasta format in Table A.4 as well as in the
241 Mendeley data repository.

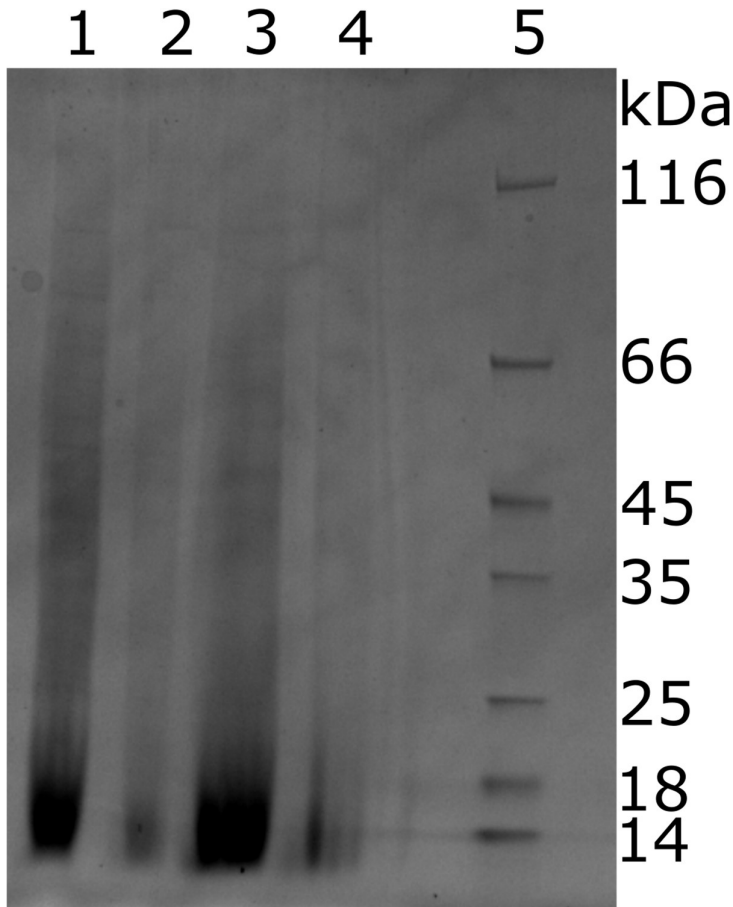
242 Although homology-inferred annotation using BLAST can indicate potential functions and localizations for
243 the individual proteins, extraction of potential functions and subcellular localization on the proteome level
244 is a tedious task. Additionally, as only verified Uniprot/Swiss-Prot proteins were included, the resulting
245 annotations were of suboptimal quality (Table A.3) due to the lack of verified annotations on related and
246 comparable species to *E. denticulatum*. Consequently, a bioinformatic prediction of subcellular localization
247 on the individual protein level was used. This data type is easily binnable for large proteomes. As the
248 DeepLoc neural network was developed for eukaryotic proteins with little or no available homology data
249 (Almagro Armenteros et al., 2017), this directly applies to the case of this study. For the entire proteome,
250 DeepLoc achieved a localization probability of 0.63 ± 0.21 (Figure A.1).

251

252 3.2. 1D SDS-PAGE analysis and protein quality assessment

253 Both protein extracts display absence of distinct proteins bands and an apparent smear along the gel
254 concentrating in the low MW range, as seen from 1D SDS-PAGE analysis in Figure 1. This is in contrast to
255 previous studies on *E. denticulatum* protein extracts (Rosni et al., 2015), where distinct protein bands were

256 observed and the low MW concentrated smear was absent. The significant difference in protein
257 appearance by SDS-PAGE may be directly ascribed to the extraction method, as the authors here used a
258 more elaborate protocol including organic (phenol) solvents as well as reducing conditions. Their approach
259 may be significantly better for efficient extraction of intact proteins from the whole seaweed, but is not
260 feasible on an industrial scale.



261
262 Figure 1: SDS-PAGE of *E. denticulatum* protein extracts investigated in this study. Protein loading is based
263 on supplied protein content of 7.1% and 70% for extract A and B, respectively. 1: Extract A, 100 µg. 2:
264 Extract A, 20 µg. 3: Extract B, 100 µg. 4: Extract B, 20 µg. 5: MW Marker.

265

266 The overall appearance of both extracts analyzed, however, are quite similar. The lack of distinct protein
267 bands could potentially indicate partial hydrolysis during extraction using high temperature under alkaline
268 conditions, as employed for both extraction methods. In addition, the extraction methodology employed
269 may also result in co-extraction of other cellular moieties, which could interfere with electrophoresis and
270 ultimately resulting in the observed smears. This has been reported for co-extracted lipids (Simões-
271 Barbosa, Santana, & Teixeira, 2000; W. Wang et al., 2004), carbohydrates (Chart & Rowe, 1991; Hashimoto
272 & Pickard, 1984), and DNA (Park, Kim, Choi, Grab, & Dumler, 2004). Further modification of proteins (e.g.
273 glycoproteins) may also add to the smearing observed on SDS-PAGE (Elliott et al., 2004; Møller & Poulsen,
274 2009; Sparbier, Koch, Kessler, Wenzel, & Kostrzewa, 2005).

275 In order to estimate the accuracy of the total protein by Kjeldahl-N analysis, we determined the soluble
276 protein content in both aqueous solution and a slightly alkaline buffer with added detergent using Qubit
277 protein assay (Table 1). From here, it is evident that the Kjeldahl-based total protein in fact correlates quite
278 well with the soluble protein content – at least when solubilized in an alkaline buffer with detergent. A
279 nitrogen-to-protein conversion factor of 6.25, the “Jones factor”, is commonly employed in food protein
280 science and has been so for 90 years (Jones, 1931; Salo-Väänänen & Koivistoinen, 1996). Nevertheless, the
281 universal conversion factor has been subject to several investigations, and species-dependent conversion
282 factors are commonly recommended (Mariotti, Tomé, & Mirand, 2008). For seaweeds in particular, the
283 factor can still vary significantly, but as no factor is available for *E. denticulatum*, a general conversion factor
284 of 5.0 can be applied (Angell, Mata, de Nys, & Paul, 2016). By doing so, and thereby lowering the protein
285 content by 20% (Table 1), the Kjeldahl-N method now underestimates the protein content compared to
286 Qubit – in particular for extract B. In this respect, it is worth considering that the conversion factor is
287 representative of the total organism proteome. Additionally, the non-protein nitrogen content of the
288 extract is undetermined, and may also influence both the Kjeldahl-N and the Qubit outputs to some degree.

289 It is also evident that the aqueous solubility of the protein in the extracts is quite low (11-15% of the total
290 protein), whereas a slightly alkaline buffer with a low amount of detergent practically fully solubilizes the
291 protein (6-fold and 10-fold solubility increase for extract A and B, respectively). This also correlates well
292 with the physical appearance of the solubilized extracts following centrifugation (Figure A.2), where a
293 significantly higher amount of solid precipitate is visible in the aqueous solutions. Nevertheless, smear and
294 apparent lack of intact high MW protein from SDS-PAGE must be taken into consideration for protein
295 quantification and in the evaluation of the protein extracts as source for further processing as well as
296 potential release of bioactive peptides.

297

Extract	Extraction method	Protein content (Kjeldahl-N * 6.25) ¹	Protein content (Kjeldahl-N * 5.0) ²	Soluble protein (ddH ₂ O)	Soluble protein (buffer)	Common contaminant proteins ³	Verified Seaweed-specific proteins ³
A	Alkaline, hot-water extraction → Ultracentrifugation → Lyophilization	7.1%	5.7%	1.1%	6.2%	20%	78%
B	Alkaline, hot-water extraction → Ultracentrifugation → Lyophilization → Acidic precipitation → Lyophilization	70%	56%	7.3%	74.8%	80%	6.0%

299 Table 1: General characteristics for the two *E. denticulatum* extracts analyzed in this work including total protein and soluble protein content. ¹Total
300 protein by Kjeldahl-N was supplied by CP Kelco. ²Calculated based on supplied protein content (¹) using a conversion factor of 5.0 (Angell et al., 2016).
301 ³Sum of relative abundance for common contaminant proteins and verified seaweed specific proteins identified in MaxQuant by I_{rel}^L for semi-specific
302 analysis with optimized parameters, prior to any filtering, but after removing trypsin (Stage 1).

303 3.3. Identification and quantification of peptides and proteins by LC-MS/MS

304 Initially, we applied an iterative process where different *in silico* digestion methods (i.e. specific, semi-
305 specific, and unspecific digestion), peptide- and protein-level FDR, and number of identified peptides per
306 protein were attempted. This was done not only to identify the optimal parameters for analysis, but also to
307 investigate the feasibility of applying the two specified quantitative metrics. The iterative process was of
308 utmost importance, as the sample quality and especially the number of identified peptides and proteins for
309 the extracts was low. A low number of peptide identifications significantly affects protein identification and
310 quantification via the impact on FDR-controlled thresholds. This is ultimately an inherent property of the
311 peptide scoring algorithm. MaxQuant employs the Andromeda search engine, in which peptide score is not
312 only based on PEP, but also on the intensity of a given feature (Cox et al., 2011; Tiwary et al., 2019;
313 Tyanova, Temu, & Cox, 2016). Consequently, high intensity features with significant PEP (i.e. potential false
314 positives), which in other studies may have been filtered out, will obtain a sufficiently high peptide score
315 and be used in protein quantification. Ultimately, this leads to false identification of proteins with a
316 significant relative abundance, which impairs further analysis. By applying more stringent thresholds on
317 both peptide and protein level, this is alleviated to some extent. Nevertheless, it may be needed to inspect
318 and evaluate PEPs rather than apply threshold filtering on peptide score alone, as PEP relies solely on PSM
319 and sequence-dependent features. This aspect is thoroughly discussed and evaluated in Appendix A.

320 By applying the optimized search parameters, a total of 66 proteins across both extracts and analysis
321 methods (tryptic and semi-specific) following filtering of trypsin and reverse hits (Stage 1) were identified
322 and quantified (Table 2). Extract B is highly contaminated since 80% (based on I_{rel}^+ for semi-tryptic analysis)
323 of all identified proteins were constituted by common contaminants (Table 1), primarily keratins. On the
324 other hand, extract A “only” contained 20%. Although common contaminants are usually filtered out prior
325 to quantification, the magnitude is noteworthy. In total, merely 40 proteins were identified across both
326 extracts and analysis conditions, following filtering of common contaminants and subsequent re-
327 quantification (Stage 2, Tables A.5; A.6). Semi-specific analysis resulted in identification of four additional

328 proteins (one in extract A and three in extract B), whereof one (c1275_g1_i1.p1) constitutes more than half
329 of the Stage 2 protein by I_{rel}^L in extract B. Furthermore, 11 proteins were not identified by this approach
330 (four in extract A, three in extract B and four identified in both extracts using tryptic conditions), but none
331 of these were of high abundance. From plotting relative abundance by both riBAQ and I_{rel}^L (Figure A.3), a
332 correlation was seen within each extract (PPC = 0.99-1.0 for extract A; PPC = 0.19-0.95 for extract B), but
333 the semi-specific analysis of extract B correlated poorly with the tryptic analysis. The correlation between
334 extracts was even worse (PPC = 0.14-0.55), indicating that the stringent quality parameters applied for
335 automatic filtering, were not fully capable of cleaning the data from bad peptide spectrum matches (PSMs)
336 and dubious protein identifications.

337

338

Stage 1								Stage 2							Stage 3						
Protein IDs	I _{rel} A tryp	riBAQ A	I _{rel} A semi	I _{rel} B tryp	riBAQ B	I _{rel} B semi	Contaminant ID	Protein IDs	I _{rel} A tryp	riBAQ A	I _{rel} A semi	I _{rel} B tryp	riBAQ B	I _{rel} B semi	Protein IDs	I _{rel} A tryp	riBAQ A	I _{rel} A semi	I _{rel} B tryp	riBAQ B	I _{rel} B semi
CON_ENSEMBL	NQ	NQ	NQ	0.1%	0.1%	NQ	Keratin	rf1c10492_g1_i1.p1	NQ	NQ	NQ	0.1%	0.1%	NQ	rf1c1505_g2_i1.p1	8.6%	11.8%	13.4%	1.2%	1.5%	1.7%
CON_O43790	NQ	NQ	NQ	0.7%	0.5%	0.6%	Keratin	rf1c1275_g1_i1.p1	NQ	NQ	NQ	NQ	NQ	11.1%	rf1c1613_g1_i1.p1	NQ	NQ	0.0%	0.0%	0.0%	0.0%
CON_P02533	0.1%	0.1%	0.0%	1.9%	1.5%	1.8%	Keratin	rf1c1294_g1_i1.p1	0.0%	0.0%	NQ	0.0%	0.0%	NQ	rf1c17304_g1_i1.p1	2.6%	2.3%	2.5%	0.3%	0.2%	0.2%
CON_P02662	NQ	NQ	NQ	0.1%	0.1%	NQ	α-S1-casein	rf1c1357_g1_i1.p1	NQ	NQ	NQ	0.0%	0.0%	NQ	rf1c17615_g1_i1.p1	0.1%	0.1%	NQ	NQ	NQ	NQ
CON_P02666	0.0%	0.0%	0.0%	2.2%	3.8%	1.8%	β-casein	rf1c17161_g1_i1.p1	NQ	NQ	NQ	0.1%	0.0%	NQ	rf1c231_g1_i1.p1	0.1%	0.1%	NQ	NQ	NQ	NQ
CON_P02754	6.5%	4.7%	6.9%	5.2%	3.7%	4.9%	β-lactoglobulin	rf1c17201_g1_i1.p1	0.0%	0.0%	NQ	NQ	NQ	NQ	rf1c2364_g1_i1.p1	0.1%	0.1%	0.1%	0.5%	0.4%	0.4%
CON_P02768	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	Albumin	rf1c17231_g1_i1.p1	0.0%	0.0%	0.0%	0.3%	0.3%	0.2%	rf1c2556_g1_i1.p1	0.0%	0.0%	NQ	0.0%	0.0%	NQ
CON_P02769	0.0%	0.0%	0.0%	1.5%	1.1%	1.3%	Albumin	rf1c2788_g1_i1.p1	NQ	NQ	NQ	NQ	NQ	0.2%	rf1c3760_g1_i1.p1	NQ	NQ	0.7%	NQ	NQ	NQ
CON_P04264	5.5%	5.6%	4.6%	33.3%	33.1%	26.7%	Keratin	rf1c3249_g1_i1.p1	0.0%	0.0%	NQ	NQ	NQ	NQ	rf1c4090_g1_i1.p1	0.1%	0.1%	0.1%	0.0%	0.0%	NQ
CON_P08779	0.0%	0.0%	0.0%	0.3%	0.2%	0.2%	Keratin	rf1c4757_g1_i1.p1	NQ	NQ	NQ	0.1%	0.1%	0.2%	rf1c4354_g1_i1.p1	3.3%	4.1%	3.1%	0.0%	0.0%	0.0%
CON_P13645	6.2%	6.9%	5.5%	19.3%	21.1%	16.8%	Keratin	rf1c4921_g1_i1.p1	NQ	NQ	NQ	0.0%	0.0%	0.1%	rf1c4671_g1_i1.p2	0.3%	0.4%	0.2%	NQ	NQ	NQ
CON_P13647	0.2%	0.2%	0.2%	1.8%	1.6%	0.9%	Keratin	rf1c5168_g1_i1.p1	NQ	NQ	NQ	0.0%	0.0%	0.1%	rf1c5232_g1_i1.p1	0.9%	0.9%	0.8%	NQ	NQ	NQ
CON_P19013	NQ	NQ	0.0%	0.2%	0.1%	0.1%	Keratin	rf1c5952_g1_i1.p1	NQ	NQ	NQ	NQ	NQ	0.1%	rf1c6313_g1_i1.p1	27.2%	25.3%	25.2%	0.2%	0.2%	1.4%
CON_P35527	1.1%	1.3%	1.1%	17.2%	19.7%	16.2%	Keratin	rf1c6797_g1_i1.p1	NQ	NQ	NQ	0.0%	0.0%	0.0%	rf1c6373_g1_i1.p1	0.0%	0.0%	0.0%	0.0%	0.0%	NQ
CON_P35908	2.3%	1.8%	1.9%	6.1%	4.7%	5.9%	Keratin	rf1c6825_g1_i1.p3	1.4%	0.9%	1.3%	2.8%	1.7%	2.1%	rf1c6458_g1_i1.p1	1.1%	1.8%	1.4%	NQ	NQ	NQ
CON_P48668	0.0%	0.0%	0.0%	0.6%	0.6%	1.3%	Keratin	rf1c6945_g1_i1.p2	0.0%	0.0%	0.0%	0.2%	0.2%	0.2%	rf1c6656_g1_i1.p1	0.0%	0.0%	0.0%	0.3%	0.3%	0.3%
CON_P78386	0.0%	0.0%	NQ	0.1%	0.1%	0.1%	Keratin	rf1c8389_g1_i1.p1	0.0%	0.0%	NQ	0.1%	0.0%	NQ	rf1c6834_g1_i1.p3	0.0%	0.0%	NQ	0.0%	0.0%	NQ
CON_Q04695	NQ	NQ	0.0%	0.2%	0.1%	0.2%	Keratin								rf1c6963_g2_i1.p1	0.1%	0.1%	0.0%	0.0%	0.0%	0.0%
CON_Q14525	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%	Keratin								rf1c7052_g1_i1.p1	27.0%	25.1%	25.8%	1.6%	1.5%	1.5%
CON_Q5D862	0.0%	0.0%	NQ	0.0%	0.0%	NQ	Filaggrin-2								rf1c7052_g1_i2.p1	3.5%	3.9%	3.2%	0.4%	0.4%	0.4%
CON_Q6KB66	NQ	NQ	NQ	0.0%	0.0%	NQ	Keratin								rf1c7216_g1_i1.p1	0.3%	0.3%	0.4%	NQ	NQ	NQ
CON_Q9UE12	0.0%	0.0%	NQ	0.6%	0.5%	0.5%	Keratin								rf1c8421_g1_i1.p1	1.2%	2.0%	1.2%	NQ	NQ	NQ
CON_Q9NSB2	NQ	NQ	NQ	0.1%	0.0%	0.0%	Keratin								rf1c926_g1_i1.p1	0.0%	0.0%	0.0%	NQ	NQ	NQ
CON_Q7Z3Y8	NQ	NQ	NQ	0.0%	0.0%	0.0%	Keratin														
CON_Q86YZ3	NQ	NQ	NQ	0.0%	0.0%	0.0%	Hornerin														
CON_Q8IUT8	NQ	NQ	NQ	NQ	NQ	NQ	Keratin														

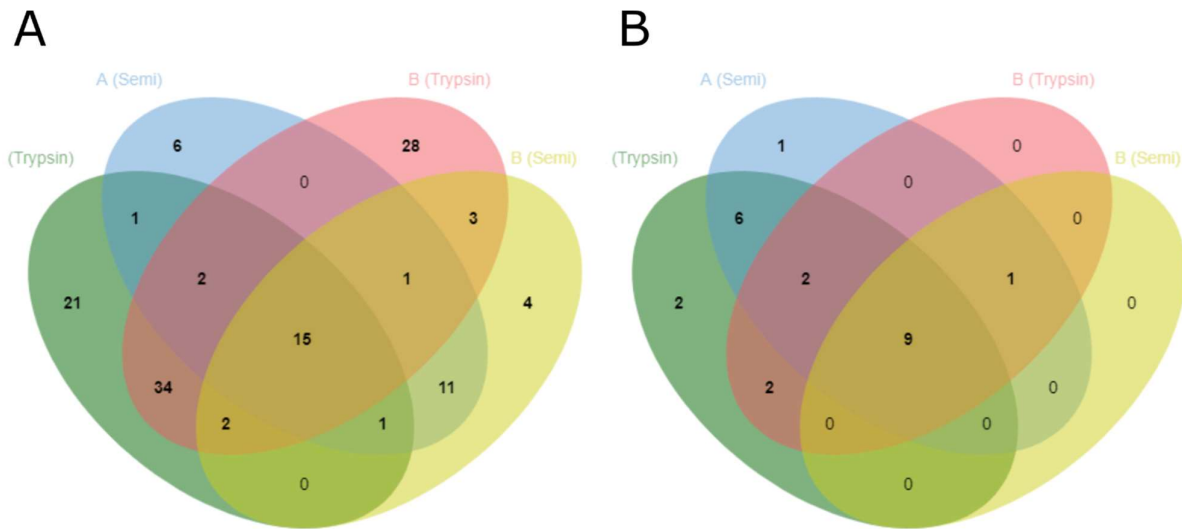
340 Table 2: Relative protein abundance of *E. denticulatum* extracts A and B (after filtering of trypsin) following
341 initial quantification (Stage 1) with optimized search parameters by I_{rel}^L and riBAQ for both tryptic and semi-
342 specific analysis. Proteins are divided in common contaminants (Stage 1 filtered proteins), false positive
343 identifications/contaminants (Stage 2 filtered protein), and final, verified proteins (Stage 3). Common
344 contaminants are annotated using their UniProt accession number. NQ: Protein not quantified in the
345 specific sample using the specific analysis method.

346

347 Identified “outliers” (Tables A.5; A.6) that did not correlate between extracts (i.e. are suddenly highly
348 enriched in extract B) may in fact be contaminants with some homology to the *E. denticulatum* proteome
349 (further details are presented in the Appendix A). For instance, in the tryptic analysis of extract B,
350 c6825_g1_i1.p3 is highly abundant but only identified by two peptides, which both map to histones from
351 e.g. humans. Histone was also the BLASTX target (*Xenopus laevis* (African clawed frog) histone H2AX) as
352 well as the predicted function by Pfam (Table A.3). Consequently, and because it was very low abundance
353 in extract A, this was ascribed as contaminant to the extract and not originating from the seaweed.
354 Although histones were bound to be identified in *E. denticulatum*, homologues from other organisms
355 would bias quantification and it was consequently excluded. Furthermore, the highly abundant protein
356 identified by semi-specific analysis of extract B only (c1275_g1_i1.p1), was also identified by only two
357 peptides. As the protein score of 11.8 was very low (see Appendix A and Table A.6), and the posterior error
358 probability (PEP) was significant (PEP>0.05), these were regarded bad PSMs and the protein ID was deemed
359 false positive. Based on these observations, manual inspection and validation was performed in order to
360 apply a final filtering step using the rationale described above. In the filtering, significant weight was put on
361 evaluation of PEP rather than peptide score, as low scoring peptides (< 40) were pre-filtered in the
362 optimized search parameters (see Appendix A for further details). Filtered proteins, along with the rationale
363 for their exclusion, can be found in Table A.7 and proteins are listed under Stage 2 in Table 2.

364 Following filtering, verified proteins were re-quantified (Stage 3) the list of identified proteins was reduced
365 from 40 to 23 proteins across extracts and conditions (Table 3). The stringent parameters applied in data
366 analysis, as well requirements for inclusion in the final list, fully alleviated the problem of new and
367 significantly abundant proteins showing up in extract B (see Table 2 and Figure A.3), as no proteins
368 exclusive for extract B, were observed (Figure 2B). Nine proteins were observed exclusively in extract A, but
369 this may be explained as loss during the extended processing for extract B. Extended processing may also
370 be a likely explanation for the extract B exclusive peptides identified (Figure 2A). Furthermore, all nine
371 proteins are of somewhat low abundance ($I_{rel}^L < 2\%$), and do not affect the overall protein distribution
372 significantly. Interestingly, the 9 proteins identified in both extracts using both analyses approaches,
373 constituted > 93% of the verified protein in extract A and > 99% of the verified protein in extract B (by I_{rel}^L).
374 In fact, three proteins (c6313_g1_i1.p1, c7052_g1_i1.p1, and c1505_g2_i1.p1) constitute more than 75% of
375 the total protein identified in both extracts (Table 3). Furthermore, an isoform of c7052_g1_i1
376 (c7052_g1_i2), which only differs in the C-terminal region of the protein, was also identified in significant
377 abundance. If included, the proteins constitute > 80% of the verified seaweed-specific protein in both
378 extracts. With MW in the range 16-24 kDa, all three (four) proteins correlated well with the observations
379 from SDS-PAGE (Figure 1), even though no clear protein bands were observed. This indicated that these
380 three (four) proteins in particular may be of certain interest as potential sources of e.g. bioactive peptides.
381 Protein sequences and experimental sequence coverage for the three major proteins are shown in Figure 3.
382 From BlastP against verified proteins in UniProtKB/Swiss-Prot (Table S3), c7052_g1_i1.p1 (as well as the
383 isoform) shows some homology to an immunogenic protein from *Brucella suis* (UniProt AC# P0A3U9),
384 whereas Pfam indicates it could be related to the DNA repair protein REV1. Neither c6313_g1_i1.p1 nor
385 c1505_g2_i1.p1 matched any proteins from the Blast homology or Pfam protein families. Consequently, the
386 nature, structure, and function of the three highly abundant proteins remains unknown.
387

388



389

390 Figure 2: 4-way Venn diagrams showing identified peptides (A) and proteins (B) with optimized parameters
391 (5% FDR and minimum score threshold) and following filtering for extract A using tryptic analysis (green),
392 extract A using semi-tryptic analysis (blue), extract B using tryptic analysis (red), and extract B using semi-
393 tryptic analysis (yellow). List sizes (in the same order) for peptides (A) are 76, 37, 85, and 37 for a total of
394 129 identified peptides. List sizes (in the same order) for proteins (B) are 21, 19, 14, and 10 for a total of 23
395 identified proteins.

396

397

Protein ID	MW [kDa]	#Pep A tryp	#Pep B tryp	#Pep A semi	#Pep B semi	Seq. cov. A tryp [%]	Seq. cov. B tryp [%]	Seq. cov. A semi [%]	Seq. cov. B semi [%]	Score tryp	Score semi	riBAQ A tryp	I _{rel} ^L A tryp	I _{rel} ^L A semi	riBAQ B tryp	I _{rel} ^L B tryp	I _{rel} ^L B semi	rTPM	Subcellular localization ¹	Subcell score ¹
c6313_g1_i1.p1	21.153	7	1	13	5	36.3	7.4	47.9	17.4	323.3	323.3	32.3%	35.5%	32.1%	3.4%	3.8%	23.3%	0.29%	Extracellular	0.6985
c7052_g1_i1.p1	24.213	7	8	16	7	36.1	34.8	45.4	31.7	323.3	323.3	31.9%	35.2%	33.0%	31.9%	35.9%	25.1%	0.02%	Extracellular	0.9128
c1505_g2_i1.p1	15.778	4	4	7	5	30	30	30	30	323.3	323.3	15.1%	11.3%	17.1%	33.4%	25.4%	28.0%	0.14%	Extracellular	0.4441
c4354_g1_i1.p1	40.332	8	1	7	3	24.6	3.5	21.7	5.6	323.3	323.3	5.2%	4.3%	4.0%	0.2%	0.1%	0.3%	0.15%	Extracellular	0.8483
c7052_g1_i2.p1	23.965	6	5	15	5	30.8	25.1	40.1	25.1	163.3	140.2	4.9%	4.5%	4.1%	8.3%	7.8%	6.8%	0.06%	Extracellular	0.9431
c17304_g1_i1.p1	27.965	6	2	6	1	23	6.7	19.7	3	188.3	190.4	2.9%	3.4%	3.2%	4.8%	5.7%	3.5%	1.08%	Extracellular	0.5089
c8421_g1_i1.p1	59.681	4	0	4	1	10	0	10	2.3	323.3	323.3	2.6%	1.6%	1.6%	0.0%	0.0%	0.0%	0.04%	Membrane	0.9998
c6458_g1_i1.p1	46.381	3	0	7	0	10.8	0	17.5	0	303.3	145.4	2.2%	1.4%	1.8%	0.0%	0.0%	0.0%	0.12%	Extracellular	0.8601
c5232_g1_i1.p1	18.952	2	0	2	0	13.5	0	13.5	0	125.3	104.9	1.2%	1.1%	1.1%	0.0%	0.0%	0.0%	0.03%	Extracellular	0.6419
c4671_g1_i1.p2	29.874	4	0	3	0	19.9	0	17	0	52.5	31.4	0.5%	0.4%	0.2%	0.0%	0.0%	0.0%	0.03%	Plastid	0.995
c7216_g1_i1.p1	25.446	2	0	3	0	10.8	0	16.9	0	16.1	19.3	0.4%	0.3%	0.4%	0.0%	0.0%	0.0%	0.08%	Extracellular	0.8121
c17615_g1_i1.p1	27.973	2	0	0	0	7.7	0	0	0	13.9	0.0	0.2%	0.2%	0.0%	0.0%	0.0%	0.0%	0.06%	Extracellular	0.9751
c6963_g2_i1.p1	165.47	10	2	4	2	6.5	1.6	2.1	1.6	108.7	55.5	0.1%	0.1%	0.1%	0.5%	0.5%	0.6%	0.04%	Plastid	0.6933
c4090_g1_i1.p1	16.129	1	2	1	1	6.8	12.9	6.8	6.1	15.3	11.2	0.1%	0.1%	0.1%	0.5%	0.7%	0.0%	0.02%	Plastid	0.9815
c231_g1_i1.p1	18.006	2	0	0	0	10.2	0	0	0	11.7	0.0	0.1%	0.1%	0.0%	0.0%	0.0%	0.0%	0.05%	Extracellular	0.9998
c2364_g1_i1.p1	50.492	1	5	2	5	2.4	9.9	4.1	9.9	135.4	105.1	0.1%	0.1%	0.1%	8.4%	10.8%	6.9%	0.23%	Cytoplasm	0.7655
c926_g1_i1.p1	79.764	3	0	2	0	3.6	0	2.6	0	20.2	10.9	0.1%	0.1%	0.1%	0.0%	0.0%	0.0%	0.04%	Extracellular	0.9924
c6373_g1_i1.p1	119.64	4	1	2	0	4.1	0.9	2.3	0	79.2	40.8	0.1%	0.0%	0.0%	0.1%	0.1%	0.0%	0.06%	Extracellular	0.5704
c6656_g1_i1.p1	43.007	3	3	1	3	8.8	8.5	3.3	8.5	79.4	75.8	0.0%	0.0%	0.0%	7.0%	7.4%	5.4%	0.12%	Plastid	0.9982
c6834_g1_i1.p3	22.388	1	1	0	0	5.3	6.7	0	0	15.3	0.0	0.0%	0.0%	0.0%	0.5%	0.7%	0.0%	0.06%	Plastid	0.9985
c2556_g1_i1.p1	57.477	1	3	0	0	2	4.9	0	0	19.4	0.0	0.0%	0.0%	0.0%	0.8%	0.8%	0.0%	0.05%	Plastid	0.567
c1613_g1_i1.p1	32.099	0	2	1	2	0	7.8	5.4	9.5	35.0	31.5	0.0%	0.0%	0.0%	0.4%	0.4%	0.2%	0.09%	Plastid	0.9671
c3760_g1_i1.p1	32.533	0	0	3	0	0	0	6.6	0	0.0	253.1	0.0%	0.0%	0.0%	0.0%	1.0%	0.0%	0.06%	Lysosome	0.3775

400 Table 3: Summary of verified proteins following parameter optimization, manual inspection, and filtering
401 (Stage 3) for *E. denticulatum* extracts A and B using both tryptic and semi-tryptic analysis. For each
402 identified protein, the molecular weight, number of identified peptides, sequence coverage, protein score,
403 riBAQ, I_{rel}^1 , rTPM, subcellular localization, and localization probability. ¹Subcellular localization and
404 localization probability was computed using DeepLoc (Almagro Armenteros et al., 2017).

405

```
>c1505_g2_i1.p1      TotalSequenceCoverage=30%  
MSHKLLPSLLLLSFLLLLNFPPPTSSTPALSPPHKNPNNALSPHLQSITEEDDDDLTA  
PSTLRRVFATPRVFNIIVRYRRFAPLALNADPETTSQIRAVLVVCLQQVRELQRDDNVRN  
VRILLSSLVLLLLDWLVLCLLN*
```

```
>c6313_g1_i1.p1      TotalSequenceCoverage=52%  
MALIWLLSIVFALLTALGTTSAVNLNPLRVNANCRNRDFPVRNNIRLRVRYVWNDMQTD  
LDTSTRFLGENVGFACSGSAQTYLSFEGDNTGRGEEVAIVEVGDARKDEAWRGTTTCIVL  
KAQWFNSRNQGNIRVIVEIRNKGTNNLIRDPLEIVARPGVGDSCSMRLIATVVVDEDEGI  
YLARAFNCPN*
```

```
>c7052_g1_i1.p1      TotalSequenceCoverage=54%  
MTPLLLLPLLLALTTANTPHPTPRSISVTGDASVAAEPDIATLTTEVVTLAPTAQAALTR  
NNRLTSALFDLSEFNVSRRDIQTTSFSVSPRFQRPDNSDVTRIIGYTVRNSLRVTVRDL  
SNLGLILDALVRAGSNLSRISFGISNEADLRDQARELAVKDAVRRATLLTKAAGTGLGK  
VLSIREGGRSTGGFSAQVRARREAEVPIAPGELQVSARVTLEIELVG*
```

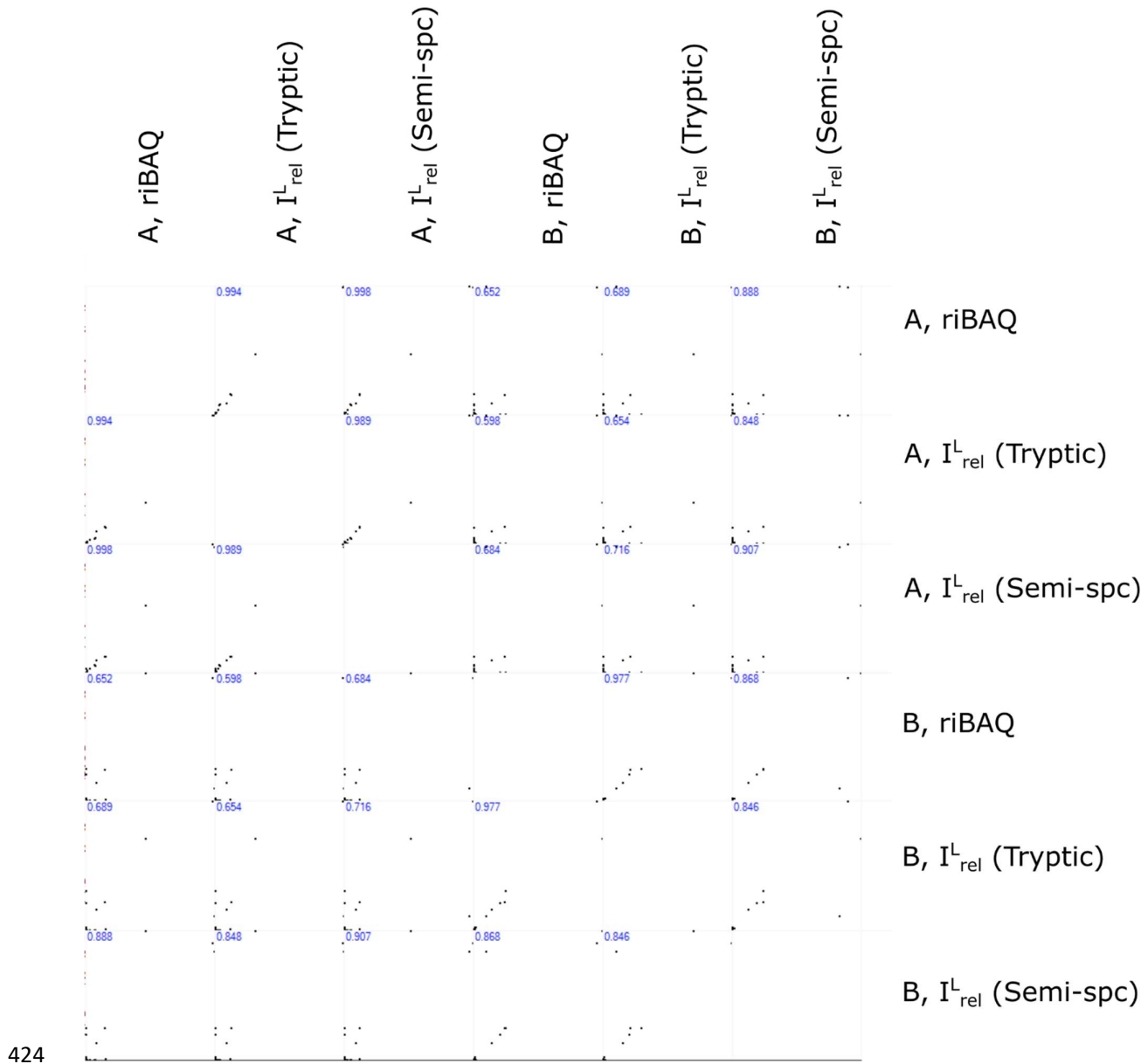
406

407 Figure 3: Protein sequence and experimental sequence coverage across both extracts and analysis methods
408 (highlighted in grey) for the three most abundant *E. denticulatum* proteins identified. All three proteins
409 passed final selection criteria (Stage 3) and accounted for 82.2% and 76.4% (quantified by I_{rel}^1 using semi-
410 specific analysis) of the verified, seaweed-specific proteins in extracts A and B, respectively. Including the
411 isoform of c7052_g1_i1 (c7052_g1_i1 – not shown), the proteins account for and 86.4% and 83.2%,
412 respectively.

413

414 Filtering resulted in improved correlation between the two extracts up to a PCC of 0.91 for relative
415 abundances quantified by I_{rel}^L (Figure 4). This indicates that in light of all the complications, the two protein
416 extracts are in fact comparable, when all redundancy and contamination was addressed. Furthermore, the
417 in-sample correlation between riBAQ and I_{rel}^L (PCC 0.87-1.0) indicated that I_{rel}^L may in fact be quite
418 powerful analogue to riBAQ for non-standard (i.e. semi- or unspecific) analysis. As semi-specific in most
419 cases increase both number of identified peptides as well as the sequence coverage on the individual
420 protein level I_{rel}^L could be a powerful tool in the analysis of proteins where partial (non-specific) hydrolysis
421 is observed, as this will include all peptide originating from the parent proteins rather than proteolytic
422 peptides alone.

423



425 Figure 4: Correlation of relative protein abundances between extracts (A and B), analysis conditions (tryptic
426 and semi-specific), and quantification method (riBAQ and I_{rel}^L) following manual validation, filtering, and re-
427 quantification (Stage 3). Pearson Correlation Coefficients are shown in blue in the upper left corner of each
428 sub-plot.

429

430 Considering the level of contamination in the extracts as outlined above, this naturally affects the potential
431 yield in targeted processing of the proteins. Including all initially identified peptides/protein including the
432 common contaminants, the final list of quantified proteins constitute 78% of the total protein for extract A
433 but merely 6.0% of the total protein for extract B. This correspond to the verified *E. denticulatum* proteins
434 (Stage 3) constituting 5.6% and 4.2% of the total extract mass, based on the total protein content for the
435 individual extracts. The observed level of contamination also indicated that although the total protein
436 content was significantly increased in extract B, this may also come at a high cost in terms of applicability.
437 However, as protein contamination can occur at all stages from processing facility to analysis lab, this
438 should be investigated further. Furthermore, the tryptic analysis showed a significantly lower number of
439 peptides and relative abundance for c6313_g1_i1.p1 compared to the semi-tryptic analysis for extract B.
440 This could indicate that this particular protein is subject to partial hydrolysis during the additional
441 processing, which again strengthens the use of the semi-specific analysis for this type of protein extract.
442 The high degree of exogenous protein identified in extract B may also explain why the N-to-protein
443 conversion factor of 5 appears to give much better results for extract A, and why extract B appears to be
444 more accurately estimated using the Jones factor of 6.25 (Table 1).

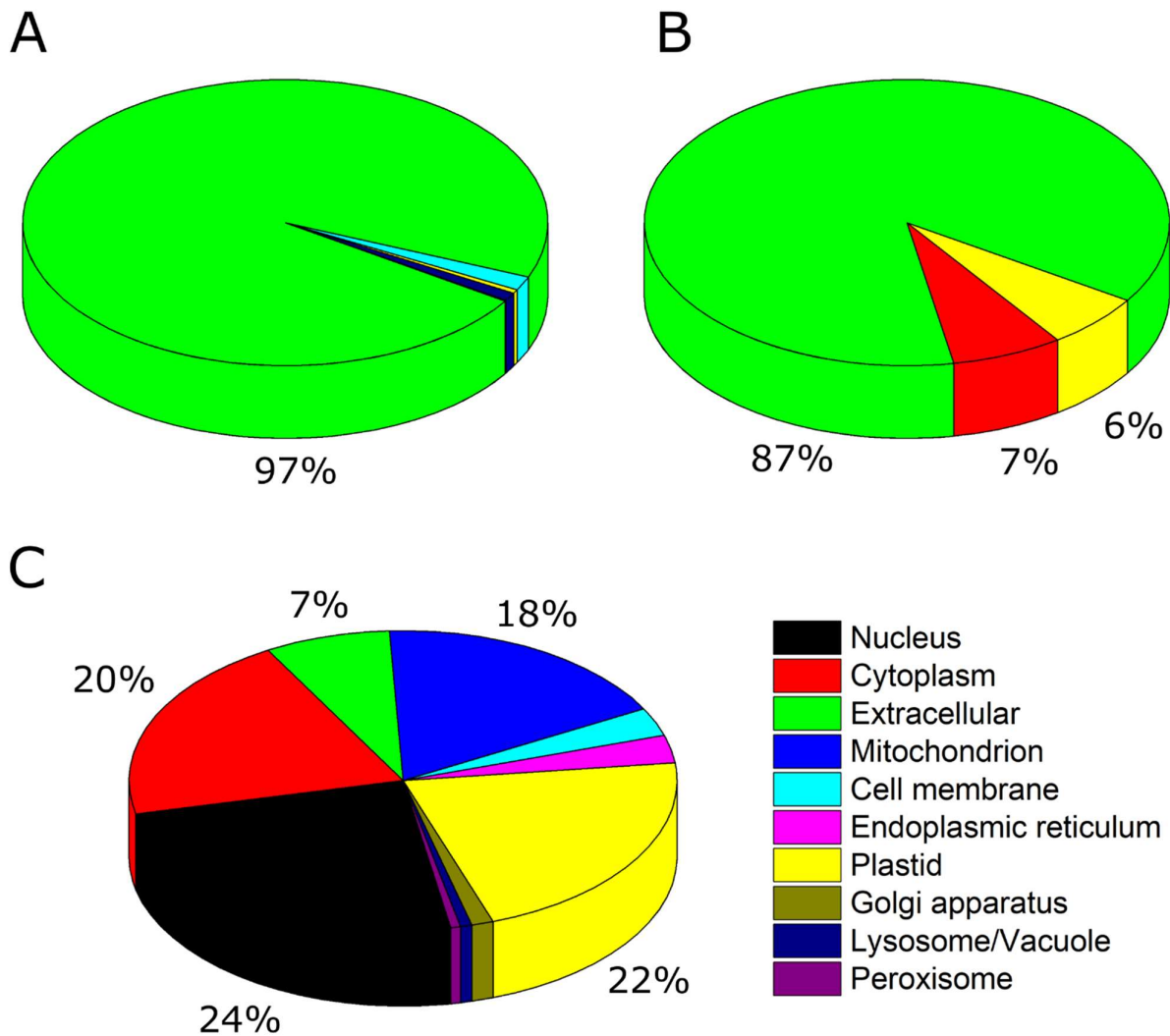
445

446 *3.4. Enrichment of extracellular proteins*

447 In Figure 5, the relative subcellular distribution of proteins predicted by DeepLoc, is presented. For the
448 transcriptome analysis (Fig 5C), a relative broad distribution of proteins (by rTPM) is observed with the
449 majority of proteins being ascribed to the nucleus (24%), plastid (22%), cytoplasm (20%), mitochondria
450 (18%), and extracellular (7%). This distribution does not correlate with the protein distribution established
451 by LC-MS/MS, regardless of data analysis conditions employed. In fact, there is a very significant
452 enrichment in extracellular proteins. For extract A (Fig 5A), almost exclusively extracellular proteins are
453 identified (97%) by I_{rel}^L . While extract B (Fig 5B) has some content of plastid and cytoplasmic protein, the

454 majority of identified proteins are extracellular (87%) by I_{rel}^L . The three primary proteins in both extracts
455 are all classified as being extracellular. Although c7052_g1_i1.p1 shows homology to a periplasmic proteins
456 by BlastP, it has a very high extracellular localization probability using DeepLoc (Table 2). At the individual
457 protein level, the extracellular protein with the highest rTPM of 1.1%, c17304_g1_i1.p1 (see Table A.3), was
458 determined to constitute 3.2-3.5% of the molar protein content. Although still significantly abundant, the
459 three highly abundant extracellular proteins described above (I_{rel}^L 17-33% each) merely constituted 0.02-
460 0.29% on the transcript level, indicating that the extraction method is not selective for extracellular
461 proteins per se, but rather a few selected extracellular proteins.

462



463

464 Figure 5: Relative subcellular protein distribution as predicted by DeepLoc (Almagro Armenteros et al.,
465 2017) for A: Protein extract A. B: Protein extract B. For both protein extracts, relative abundance was
466 estimated by I_{rel}^L through semi-tryptic analysis using optimized parameters, following manual inspection,
467 validation, and filtering. C: Transcriptome analysis (by rTPM).

468

469 The fact that extracellular protein were almost exclusively identified in the extracts, is also very likely to
470 explain the low extraction yields observed at the pilot plant (unpublished data from CP Kelco). From 20 kg

471 of seaweed, 155 g material was obtained using a 1000 L extraction tank (Extract A). The protein content (by
472 Kjeldahl-N and converted using the Jones factor) of 7.1% correspond to merely 11 g of protein following
473 extraction corresponding to a protein yield of 0.055%. Further processing to concentrate protein by acid
474 precipitation (Extract B) yielded 6.7 g of product with 71% protein corresponding to 4.8 g of protein and
475 consequently a loss of 57% protein mass and thus an even lower yield (0.024%). These findings indicate
476 that the hot-water extraction used to obtain the extracts, is not capable, to a significant degree, to disrupt
477 cells and release intracellular proteins. Low protein yields using simple aqueous extraction from *E.*
478 *denticulatum* has previously been reported in literature (Bjarnadóttir et al., 2018; Fleurence, Le Coeur,
479 Mabeau, Maurice, & Landrein, 1995). This, in turn, implies that there is still a significant potential for
480 protein extraction from the seaweed and other approaches such as for instance pressurized and
481 supercritical fluid extraction (Herrero, Sánchez-Camargo, Cifuentes, & Ibáñez, 2015), addition of cofactors
482 (Harnedy & FitzGerald, 2013a; Maehre, Edvinsen, Eilertsen, & Elvevoll, 2016), microwave-assisted
483 extraction (Magnusson et al., 2019), ultrasound-assisted extraction (Bleakley & Hayes, 2017), or any
484 combination thereof (Cermeño, Kleekayai, Amigo-Benavent, Harnedy-Rothwell, & FitzGerald, 2020), may
485 be more suitable. Enzyme assisted extraction (EAE) is an emerging technology for seaweed protein
486 extraction, showing great potential (Hardouin et al., 2016; Naseri, Marinho, Holdt, Bartela, & Jacobsen,
487 2020; Terme et al., 2020; Vásquez, Martínez, & Bernal, 2019). In a recent study, enzyme assisted extraction
488 of *E. denticulatum* increased the protein yield up to 60% using Alcalase® or Viscozyme® (0.2% w/w) at pH 7
489 and room temperature (Naseri, Jacobsen, et al., 2020). The increased protein extraction efficiency was
490 furthermore obtained without compromising the downstream carrageenan production. However, this
491 method is not at present implemented by the carrageenan industry.

492

493 **4. Conclusion:**

494 Using *de novo* transcriptome assembly, we were able to construct a novel reference proteome for
495 *E. denticulatum*, which was used to characterize two pilot-scale, hot-water extracts. Although further
496 processing (extract B) increased protein content significantly (compared to extract A), the aqueous
497 solubility of both was quite low and both extracts displayed a high degree of smear and a lack of distinct
498 protein bands by SDS-PAGE. A slightly alkaline pH and addition of a small amount of detergent fully
499 solubilized the protein. From proteomics studies, using label-free quantification of non-standard protein
500 digests via a novel length-normalized relative abundance approach, we determined that further extract
501 processing may have introduced a significant amount of contaminant proteins not originating from the
502 seaweed. After filtering of contaminant proteins and potential false-positive protein identifications, the
503 protein content from the two extracts correlated quite well. Using subcellular localization prediction, we
504 determined that both extracts were highly enriched in extracellular protein compared to the expected
505 protein distribution from quantitative transcriptome analysis and estimated protein copy number. In fact,
506 more than 75% of the seaweed-specific protein identified and quantified, was constituted by merely three
507 proteins, which were predicted to be extracellular. Extracellular protein enrichment indicates that hot-
508 water extraction is not capable of extracting intracellular proteins, but may be useful for isolation of
509 extracellular protein content on large, industrial scale. Further processing of seaweed extracts is useful for
510 increasing total protein content, but it requires further optimization to reduce the introduction of a large
511 degree of exogenous protein, the depletion of species-specific proteins, and the significant loss in total
512 protein. Nevertheless, this study illustrates the applicability of quantitative proteomics for characterization
513 of extracts to be used as potential sources of novel food protein or bioactive peptides. Furthermore, the
514 results clearly demonstrate the power of the methodology, particularly in combination with quantitative
515 transcriptomics and bioinformatics, for evaluating extraction methods and for use as a guide in the
516 development and optimization of industrial processes.

517

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521

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524

525 **7. Author Contribution**

526 S.G.: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft preparation,

527 Writing – review and editing, Visualization. M.P.: Methodology, Formal analysis, Investigation, Writing –

528 original draft preparation, Writing – review and editing. P.M.: Conceptualization, Methodology, Writing –

529 review and editing, Supervision. S.L.H.: Writing – original draft preparation, Writing – review and editing.

530 C.J.: Writing – original draft preparation, Writing – review and editing, Funding acquisition. P.J.G-M.:

531 Methodology, Writing – review and editing. E.B.H.: Conceptualization, Writing – review and editing, Project

532 administration, Funding acquisition. M.T.O.: Conceptualization, Writing – review and editing, Supervision.

533

534 **8. Conflict of Interest**

535 The authors declare no conflict of interest.

536

537 **9. Data Availability**

538 MaxQuant output files (txt folder) can be accessed through the linked Mendeley Data repository

539 (Gregersen, 2020). Raw MS data can be made available upon request.

540

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649 [eXWv&sig=edeezmYHONz_m89QRHtgI-94oGg&redir_esc=y#v=onepage&q&f=false](https://books.google.dk/books?hl=en&lr=&id=MaM4Z5IDcjoC&oi=fnd&pg=PA9&ots=QAQAi-eXWv&sig=edeezmYHONz_m89QRHtgI-94oGg&redir_esc=y#v=onepage&q&f=false)
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