Received: 15 March 2023 | Revised: 28 September 2023 | Accepted: 30 September 2023

DOI: 10.1002/app.54789

RESEARCH ARTICLE

Applied Polymer SCIENCE WILEY

Comprehensive characterization and evaluation of the process chain and products from *Euphausia superba* exocuticles to chitosan

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Funding information
Bundesministerium für Bildung und Forschung, Grant/Award Number: 031A567A; Carl-Zeiss-Stiftung, Grant/Award Number: P2019-02-004

Abstract

Antarctic krill (*Euphausia superba*) is a source for compounds of high nutritive value. Within that process of extraction, exocuticles (shells) accumulate which are currently disposed. A valorization of the compounds of the exocuticle such as chitosan would be beneficial to avoid waste and to obtain a versatile polymer at the same time. In contrast to previous investigations focusing on chitosan production from whole krill, we applied and optimized process stages of the chitosan production from the exocuticles, performing a comprehensive analytical evaluation of the whole process, the side streams and the products for the first time. Degreasing was the first step resulting in a krill oil yield of 6.2% using ethanol. The fatty acid profile exhibited high contents of phospholipids (21.2%). Citric acid offered a demineralization efficiency of 93%. Deproteinization investigation revealed 2 M NaOH and 90°C for 2.5 h to be the best parameters, resulting in a deproteinization efficiency of 99.9% and a chitin content of 92.8%. The spectroscopic investigation indicated that the chitin has a crystallinity index of 76% and an acetylation degree of 88%. The deacetylation degrees of the resulting chitosans is determined to be 74%–88%, the molecular weight ranges from 102 to 126 kDa.

KEYWORDS

Antarctic krill, characterization, chitin, chitosan, deacetylation, *Euphausia superba*, extraction

1 | INTRODUCTION

Chitosan is a nitrogen-containing polysaccharide which could be applied inter alia for wastewater purification, for biomedical purposes and in textile processes.[1–3] Due to its free primary amine groups, the application areas could potentially be widened after modification.[4,5] The production of native chitosan is based on the conversion of the high-molecular weight and highly acetylated polysaccharide chitin. In common, highly concentrated bases are used for the deacetylation of the chitin. This step is of great importance for the functionalization of the biopolymer. Due to the deacetylation process, the chitosan has a lower crystallinity than chitin, which makes it soluble in

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acidic solutions and more accessible for reagents, thus considerably increasing the number of possible applications.[6] Enzymatic methods for deacetylation suffer from low efficiency yet and are only in the future an alternative to the chemical deacetylations.[7] In contrast to that, the chitin sources are thereof less unilateral with insects, fungi, crustaceans or mollusks.[8] While the extraction from insects, fungi, and mollusks is still subject of research, the commercial extraction of chitin is particularly performed with marine fishery waste, namely shrimp and crab exocuticles.[9,10] However, there are much more marine waste streams with increased potential for chitin production, such as Antarctic krill (*Euphausia superba*, krill). Krill has a standing biomass of 342–536 million tons and is a valuable target of marine fishery since it is a rich source of high quality proteins, lipids, and minerals.[11] Within the enzymatic processing to obtain a protein hydrolysate, krill exocuticles accumulate. Currently, there is no use of krill exocuticles and they are discarded although the exocuticles are potentially a valuable source of chitin and chitosan after deacetylation.

The chitin content of (defatted) krill exocuticles was determined to be higher than 28% and thus at least on the same level as the content in other marine waste streams.[12,13] In contrast to that, there are only a few studies with regard to the chitin purification from krill. Yuan et al.[14] purified chitin, produced chitosan and compared the krill chitosan characteristics with the chitosan obtained from white shrimp and giant river prawn. An optimization process was not part of this investigation.

Independent from the marine source, the purification of the chitin typically consists of 3–4 chemical process steps: degreasing, demineralization, deproteinization and eventually bleaching together with mechanical or physical unit operations. Previous studies investigating the degreasing of the Antarctic krill focused on the whole biomass as source — rather than the exocuticles alone — yielding at maximum 21% of the biomass as krill oil. The demineralization of the marine biomass for chitin production was a must for marine sources. Therefore, hydrochloric acid is the acid of choice. However, it was shown that citric acid was a valuable alternative revealing demineralization efficiencies between 85% and 93% using other marine sources than krill. Investigations concerning the demineralization of Antarctic krill are missing, whereby quantitative analyzes to evaluate the efficiency of the demineralization are rarely carried out in general. In contrast, deproteinization of krill exocuticles was already investigated using NaOH resulting in a deproteinization efficiency of 97.5%.[12] Though, the variation of deproteinization conditions was not performed yet in order to determine the impact on the efficiency, the chitin content, yield, and the recovery. Furthermore, the krill-derived chitin was not subject of a thorough spectroscopic investigation.

In general, the identification of suitable solvents for degreasing, demineralization, and deproteinization were not in the focus of studies.[15,16] Additionally, the waste streams of the these steps are in general not subject of analytics to evaluate the potential of these streams, although potential applications of the side streams have already been postulated by Yan and Chen.[9]

The succeeding chitin conversion was commonly performed using concentrated alkali resulting in chitosan with a deacetylation degree of about 75%, an ash content lower than 1% and greatly diverse molecular weights of 157 and 1110 kDa, determined via viscometry. As with the other process steps, data to evaluate particularly the chitosan's purity and the process efficiency itself are missing.

Within this work, data concerning the whole value chain to produce chitosan from Antarctic krill (*E. superba*) including a comprehensive quantitative evaluation of the production process is shown for the first time. In a first step, we focus on chitin isolation, especially the acid selected for demineralization and the optimization of deproteinization conditions succeeded by the identification of suitable chitin conversion parameters. Yield and purity values are stated to compare the process for chitosan production with those from other sources. Additional to the main value chain, we provide data with regard to the composition of the side streams derived from chitin production in order identify suitable application fields.

2 | RESULTS AND DISCUSSION

The chitin purification is the first step to produce the chitosan. Even if the chitin's processability and applicability is constrained, it has a value in itself regarding the recent identification of more and more application fields. However, a further processing to chitosan is the royal road to valorize the Antarctic krill, wherefore it allies to the chitin production.

2.1 | Chitin purification

Three steps have to be performed to end up in a purified chitin from Antarctic krill exocuticles: Degreasing, demineralization, and deproteinization. The assessment of each step and the composition of the supernatants from degreasing and demineralization is hereafter elucidated.

2.1.1 | Degreasing evaluation

Degreasing was performed as a first chitin purification step and, at the same time, to obtain the krill exocuticle oil. The oil is mainly composed of lipids and fatty acids and can be prospectively used as dietary supplement. For extraction, different solvents were investigated, the results are shown in Table 1.

Highest yield of krill oil was obtained using an extraction based on ethanol with 6.2 ± 0.1% in relation to the dry krill exocuticle. Considering the amount of krill oil extracted, additional 0.4%–2.2% of biomass was lost. Comparable high yields were also achieved with a mixture of acetone and ethanol while the extraction using acetone or n-hexane resulted in poor krill oil yields. Especially Soxhlet extraction with n-hexane — which is a known method to determine fatty acid content — yielded only 58.1 ± 0.4% of the amount that could be isolated with ethanol application. As n-hexane is a non-polar solvent, its solving power related to polar lipids is limited just like those of acetone.

Literature comparison of krill oil yield and composition is only possible to a limited extent, since the oil in these publications is commonly obtained from the whole Antarctic krill, not only from the exocuticles like in this work. Ethanol always revealed the highest krill oil yield in solvent comparative studies, ranging from 9% to 21%, because of its high solubilizing properties for the phospholipids.[17–19] These extraction yields are substantially higher than those obtained in the present study (5.7%) because of the different substrates (exocuticle vs. whole). In these studies different solvents were also tested and overall determined a less efficient extraction of krill oil with acetone (5%–9%) and n-hexane (5%–12%). An acetone/ethanol mixture extracted 12% krill oil of whole frozen Antarctic krill according to the study of Gigliotti et al.[20] In our study, krill oil yields are with 5.7% slightly lower, which probably results from the fact that only the exocuticles were used to extract the oil and not the complete krill.

Antarctic krill is extremely rich in phospholipids with a content commonly found between 20% and 36%, which makes it unique compared to other Crustacea.[20,21] In addition, the phospholipids contain major parts of the polyunsaturated fatty acids (PUFA), especially the omega-3 fatty acids, eicosapentaenoic (EPA) acid, and docosahexaenoic acid (DHA).[19,22] Polar solvents like ethanol have strong solubilizing properties for polar lipids like phospholipids, resulting in a high phospholipid amount in this study (21.2 g/100 g).[23] Therefore, the PUFA content is also increased in the obtained oil by ethanol extraction (see Table 1, column 3). In our study higher contents of neutral lipids (78.3%) and cholesterol (9%) were determined when using n-hexane in comparison to polar solvents like ethanol or acetone. Nonpolar solvents, like n-hexane, lead to a very low phospholipid content in the krill oils and thus to a low PUFA content, in contrast to neutral lipids and cholesterol.[17] Gigliotti et al.[20] determined cholesterol contents of 2.5%–5.5% for extractions with acetone, ethanol, and acetone/ethanol, which are comparable to our results with the same solvents (3.6%–7.3%). Since cholesterol is associated with cardiovascular diseases, only low amounts should be present in the krill oil.[24]

Since ethanol resulted in the highest yield and a promising phospholipid content of 21.2 g/100 g, is not toxic and does not require a mixture of different solvents, it was integrated in the process as standard solvent for degreasing of the krill exocuticles previous to the steps of demineralization and deproteinization. The krill oil extracted in this study with ethanol contained high amounts of phospholipids and therefore PUFA, and at

TABLE 1 Lipid yield, phospholipid, cholesterol, and fatty acid contents of krill oil.

Solvent	n-Hexane	Ethanol	Acetone/ethanol	Acetone
Krill oil yield (%)	3.6 ± 0.2	6.2 ± 0.1	5.7 ± 0.3	2.8 ± 0.1
Biomass loss (%)	4.0 ± 0.2	8.4 ± 0.5	6.7 ± 0.6	4.0 ± 0.6
Total Lipids (g/100 g)	79.3	60.7	67.2	66.1
Neutral lipids (g/100 g)	78.3	39.5	46.1	64.5
Phospholipids (g/100 g)	<1	21.2	21.2	1.6
Cholesterol (g/100 g)	9.0	3.6	4.0	7.3
EPA[a] (C20:5) (g/100 g)	5.5	10.2	11.5	11.0
DHA[b] (C22:6) (g/100 g)	3.2	5.8	6.5	6.3
Total PUFA[c] (n-3) (g/100 g)	11.1	18.2	20.1	20.1

Note: Extraction with different solvents in comparison with the established n-hexane Soxhlet extraction.
[a]Eicosapentaenoic acid.
[b]Docosahexaenoic acid.
[c]Polyunsaturated fatty acids.

the same time low amounts of cholesterol, which makes it suitable as dietary supplement for benefitting health.[20]

2.1.2 | Demineralization evaluation

The degreased krill exocuticles were subjected to demineralization investigating different acids. Demineralization is mandatory since the mineral content of the raw krill exocuticles amounts to 32.5% in relation to the dry weight. Hydrochloric, formic, citric, and malic acid were compared as demineralization agent, water-induced mineral removal was carried out as a blank but did not result in a significant mineral removal. Demineralization efficiency was evaluated based on the final ash content of the intermediate products.

As can be seen in Figure 1, demineralization efficiencies resulted in values up to 95.9 ± 1.2% (hydrochloric acid), meaning a final mineral content of 1.3 ± 0.4%. Formic acid and malic acid gave efficiencies of 88.2 ± 1.4% and 89.0 ± 0.2%, respectively.

With citric acid a final ash content of 2.5 ± 0.1% could be reached and hence a demineralization efficiency of 92.3 ± 0.3% which is approximately the same value obtained with hydrochloric acid. Therefore, citric acid is the best alternative to substitute the corrosive agent hydrochloric acid. However, prospective analyzes will reveal if the detrimental effects on the intrinsic properties, including reduced molecular weight and degree of acetylation of purified chitin caused by hydrochloric acid can be avoided using citric acid instead.[15,25] To further reduce mineral content after demineralization with citric acid, an approach with heating the demineralization suspension to 75°C for 2 h was conducted according to El Knidri et al.[16] The mineral content in the residual biomass was 2.2 ± 0.4%, resulting in a demineralization efficiency of 93.0 ± 1.1%. The biomass loss amounted to 44.8 ± 2.9%. Since this approach did not reveal any significant improvement, the process is maintained at room temperature incubating the raw material overnight in a citric acid solution.

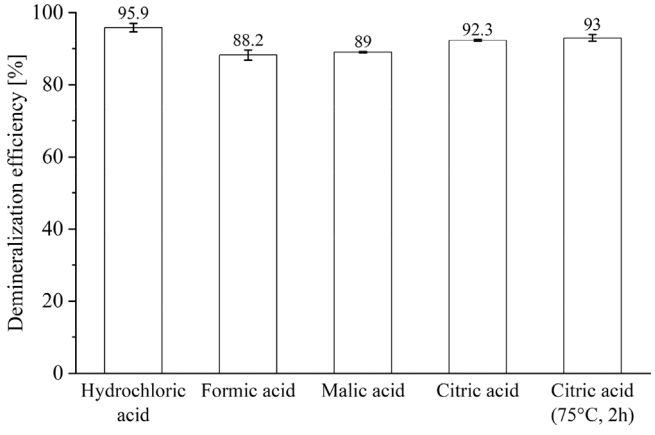

FIGURE 1 Demineralization efficiency obtained after demineralization of degreased krill exocuticles with different acids.

Demineralization occurs when different acids react with the calcium carbonate—which is the main mineral in shrimp exocuticles and presumably also in krill exocuticles—to neutralize the acid and produce soluble salts that can be washed out.[25,26] A comparison of the results with data from other studies is limited, since the ash content is usually only given for the end products of the value chain, chitin and chitosan. This limits a direct comparison with the demineralization efficiencies achieved here. Kjartansson et al.[26] determined a residual mineral content of 2.5 ± 0.1% after demineralization of North Atlantic shrimp exocuticles (*Pandalus borealis*) with a low hydrochloric acid concentration of 0.25 M. The results we obtained with 1 M citric acid are similar and it is even possible to reduce the citric acid concentration without major efficiency losses: Charoenvuttitham et al.[15] decreased the initial ash content of Black tiger shrimp exocuticles of 31.2% with 0.25 M citric acid by 85.8%. This value is only slightly lower regarding our results. Percot et al.[25] confirmed within their kinetic study that—with a solid/liquid ratio of 1/40 and 0.25 M hydrochloric acid—an incubation time of about 15 min is sufficient for demineralization. This indicates that further optimization investigations are required making the process more economic and ecologic.

2.1.3 | Composition of the demineralization supernatant

To further determine the composition, the supernatant after citric acid-assisted demineralization was freeze-dried and analyzed. A major portion (88.6%) of the supernatant is the citric acid used, the elements determined were calcium (36,000 mg/kg), potassium (270 mg/kg), iron (24 mg/kg), phosphorus (17,000 mg/kg), and magnesium (6300 mg/kg). The organic salts formed during demineralization could be used in industry. Calcium citrate is a food additive, also known as E333. Magnesium and phosphorus are involved in bone health and thus making them usable in remedies for bone loss. Iron can also be used as supplement for persons suffering from iron deficiency.[27]

2.1.4 | Deproteinization optimization

After demineralization with citric acid, the residual krill exocuticles were subjected to deproteinization with NaOH under various conditions to identify optimal

conditions. After an extensive literature research, different conditions with temperatures less than 90°C and alkali concentrations less than or equal to 2 M were chosen. The aim is to remove a maximum quantity of proteins within this step. The data achieved is shown in Figure 2.

Biomass loss for the deproteinization conditions 1–4 is in a range between 53% and 62%. Deproteinization carried out with 2 M NaOH at 90°C for 2.5 h (DP 1) resulted in the highest deproteinization efficiency of 99.9 ± 2.3%. The efficiencies obtained at milder conditions resulted in slightly lower efficiencies but are in a comparable range (88.9%–92.2%). Although deproteinization at room temperature for 24 h differs significantly from DP 1, it does not significantly differ from DP 2 and DP 3. The chitin content in the resulting deproteinized biomasses is 93.0 ± 1.1%, 88.7 ± 1.4%, 92.9 ± 0.3%, and 89.9 ± 0.3% of dry weight after DP 1–4, respectively. A reutilization of the deproteinization solution is constrained due to the high temperature and concentration of alkali applied.

Wang et al.[12] reduced the protein content in exocuticles of Antarctic krill from 47.6% to 1.2%, which represents a deproteinization efficiency of approximately 97.5%, with 2.5 M NaOH at 75°C for 1 h. They also visualized the krill exocuticles with SEM analysis after deproteinization showing that the chitin structure of tightly arranged fibers was still present. Percot et al.[25] verified that during deproteinization of exocuticles of marine shrimps with 1 M NaOH at room temperature the amount of proteins released in the supernatant increased while, at the same time, the protein content in the solid

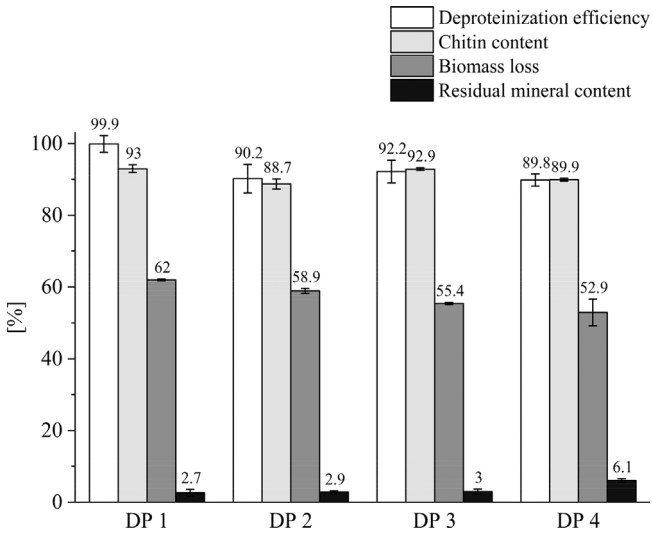

FIGURE 2 Deproteinization data concerning efficiency, biomass loss, and mineral content. DP 1: 2 M NaOH, 90°C, 2.5 h; DP 2: 1 M NaOH, 80°C, 3 h; DP 3: 2 M NaOH, 70°C, 4 h; DP 4: 2 M NaOH, RT, 24 h.

decreased. After 6 h the reaction was considered to be complete as the residual protein content was below 2%. In our study, only the condition of DP 1 with 0.7 ± 0.3% led to a protein content of less than 2%, while applying the conditions of DP 2, DP 3, and DP 4, the chitin still contained 4.2 ± 1.6%, 2.4 ± 0.1%, and 4.7 ± 1.6% residual proteins, respectively. Deproteinization at room temperature with an even higher alkali concentration (DP 4) resulted in slightly lower efficiency in this research. However, milder conditions prevented the hydrolysis of the chitin. Percot et al.[25] did not observe the hydrolysis of chitin performing the deproteinization even at 70°C. In comparison to the drastic conditions of the subsequent deacetylation, hydrolysis and deacetylation during deproteinization are likely to be negligible. As is the case for the extraction temperature, the concentration of the alkali in the solution has also a significant impact on the deproteinization efficiency. In the present study, there is no valid difference recognizable and the effect of the NaOH concentration could not be unraveled since the other parameters were also varied.

2.1.5 | Chitin content and process characteristics after deproteinization

After deproteinization a chitin with a low quantity of impurities was obtained. There is a residual mineral content in the chitin with 2.7 ± 0.9% to 6.1 ± 0.5%. Preferably, chitin or at least the deacetylated form of it, chitosan, should contain less than 1% of minerals for being high quality.[28,29] The increase in mineral content in the chitin after alkali treatment is due to the removal of proteins, a reduction of the total biomass and thus an increased share of the minerals from the total biomass. Rojsitthisak et al.[30] confirmed that during deacetylation of chitin an additional removal of minerals can be observed. Therefore, the purification during the deacetylation step of the chitin may lower the mineral content in the final chitosan.

Additionally, a change in the process sequence with prior deproteinization before demineralization should be considered to further reduce mineral content. Rojsitthisak et al.[30] hypothesized, if deproteinization is carried out first, the protective layer of proteins on minerals is removed, and the residual unshielded biomass is exposed to the acid during demineralization, leading to higher efficiencies.

The product of deproteinization contained at least 88% chitin. To further assess the deproteinization conditions, the chitin yield in relation to the applied krill exocuticle biomass and the chitin recovery in relation to the chitin content measured by acid detergent fiber (ADF)

TABLE 2 Chitin yield and chitin recovery after degreasing with ethanol, demineralization with citric acid, and the deproteinization conditions of DP 1–4.

	DP efficiency (%)	Chitin content (%)	Chitin yield[a] (%)	Chitin recovery[b] (%)
DP 1	99.9 ± 2.3	93.0 ± 1.1	15.8 ± 1.9	79.6 ± 9.4
DP 2	90.2 ± 4	88.7 ± 1.4	19.6 ± 2.0	94.4 ± 4.0
DP 3	92.2 ± 3.2	92.8 ± 0.3	20.7 ± 0.2	100.0
DP 4	89.8 ± 1.7	89.9 ± 0.3	21.2 ± 1.7	100.0

Note: Due to the deviations within the method used, recoveries slightly above 100% were measured in some cases and are here displayed as 100%.
[a]Regarding total krill exocuticle mass.
[b]Regarding chitin content in total krill mass.

respectively acid detergent lignin (ADL) analysis at the beginning (19.8%) were calculated. The values of the different deproteinization conditions DP 1–4 can be taken from Table 2. The yields calculated for DP 3 and DP 4 even exceed the chitin content of the raw material. It can be expected that these deviations are in the frame of the error margins of the measuring method. The overall chitin recovery was at least 80% and even reached 100% for the least drastic deproteinization at room temperature. That means, there is a chitin loss of less than 20% for all deproteinizations.

The chitin yield extracted from shrimp waste is describe to be in a range between 13% and 20%.[26,28,30] The yields in our research are in the same range, from 15.8% to 21.2%. It is noteworthy to say that our yield is calculated with regard to the real chitin content via ADF/ADL analysis in the deproteinized, rather than the complete biomass.

According to Kjartansson et al.,[26] an acceptable residual protein content in extracted chitin is lower than 3.5%. This was achieved by applying the conditions of DP 3 and DP 1. DP 1 showed the highest deproteinization efficiency, a high chitin content in residual biomass and at the same time the lowest residual mineral content, and was hence selected for the production of larger amounts of biomass for deacetylation trials.

2.1.6 | Overall evaluation of the chitin production process

The overall composition of krill exocuticles is derived from the different analyzes regarding lipid/fat content, ash content, protein content and chitin content. The chemical composition of the raw material is shown in the following Table 3.

The major contaminants of the krill raw material consist of ash, proteins, and to a minor share of lipids. In addition, there is a residual share of 3.7%, which could not be identified. The experiments conducted previously to optimize the individual steps of the chitin purification from krill exocuticles resulted in the following complete process: (1) degreasing of krill exocuticles with ethanol at room temperature overnight, (2) demineralization of degreased krill exocuticles with 1 M citric acid at room temperature overnight, and (3) deproteinization of degreased and demineralized krill exocuticles with 2 M NaOH at 90°C for 2.5 h. Every purification step resulted in a biomass/contaminant removal because of the extraction of the desired components, lipids, minerals, and proteins, with a yield of extraction of 172.2 ± 0.1%, 92.3 ± 0.3%, and 98.3 ± 0.7%, respectively. As already mentioned, the extraction of lipids is more efficient with ethanol than with n-hexane, resulting in a yield of over 100%. Considering the conditions of the overall process for chitin preparation presented here, the following biomass losses resulted: 8.4 ± 0.5%, 44.9 ± 3.0%, and 62.0 ± 0.3% for degreasing, demineralization, and deproteinization, respectively.

The chitin content in the respective biomass significantly increases after each process step from 19.8 ± 0.1% in the original krill exocuticles to 93.0 ± 1.1% in the biomass after deproteinization. The biomass still contains 0.7 ± 0.3% proteins and 2.7 ± 0.9% minerals, and therefore still an undefined impurity of approximately 4%. Anyway, chitin yield is 17.9 ± 1.9% and chitin recovery 90.2 ± 9.4%. The chitin obtained in this process was successfully deacetylated.

2.1.7 | Spectroscopic characterization of krill exocuticles and the quality of the purified α-chitin

The structural properties and the characteristics of the chitin were analyzed in order to evaluate the purification process and the source, compared to other natural chitin resources and the DSP developed. The results of the analyzes are shown in Figure 3.

Solid state nuclear magnetic resonance spectroscopy (in particular ^{1}H-^{13}C CP MAS NMR) is a method widely

TABLE 3 Composition of the raw material, the krill exocuticles, and the processed material (after DP1) on a dry matter basis (93.5%).

	Original raw material	Degreasing	Demineralization	Processed material
Ash content (%)	32.5 ± 0.2	-	2.5 ± 0.1	2.7 ± 0.8
Protein content (%)	40.3 ± 0.1	41.4 ± 0.1	46.7 ± 0.6	0.7 ± 0.3
Lipid content (%)	3.6 ± 0.2	0	0	0
Chitin content (%)	19.8 ± 0.1	20.9 ± 0.2	53.1 ± 1.4	93.0 ± 1.0
Compounds identified (%)	96.3 ± 0.6	-	102.3 ± 2.1	96.4 ± 2.1
Total biomass (%)	100	91.6 ± 0.5	50.5 ± 3.0	19.2 ± 0.3
Biomass loss per step (%)	0	8.4 ± 0.5	44.9 ± 3.0	62.0 ± 0.3

Note: - stands for measurements not performed.

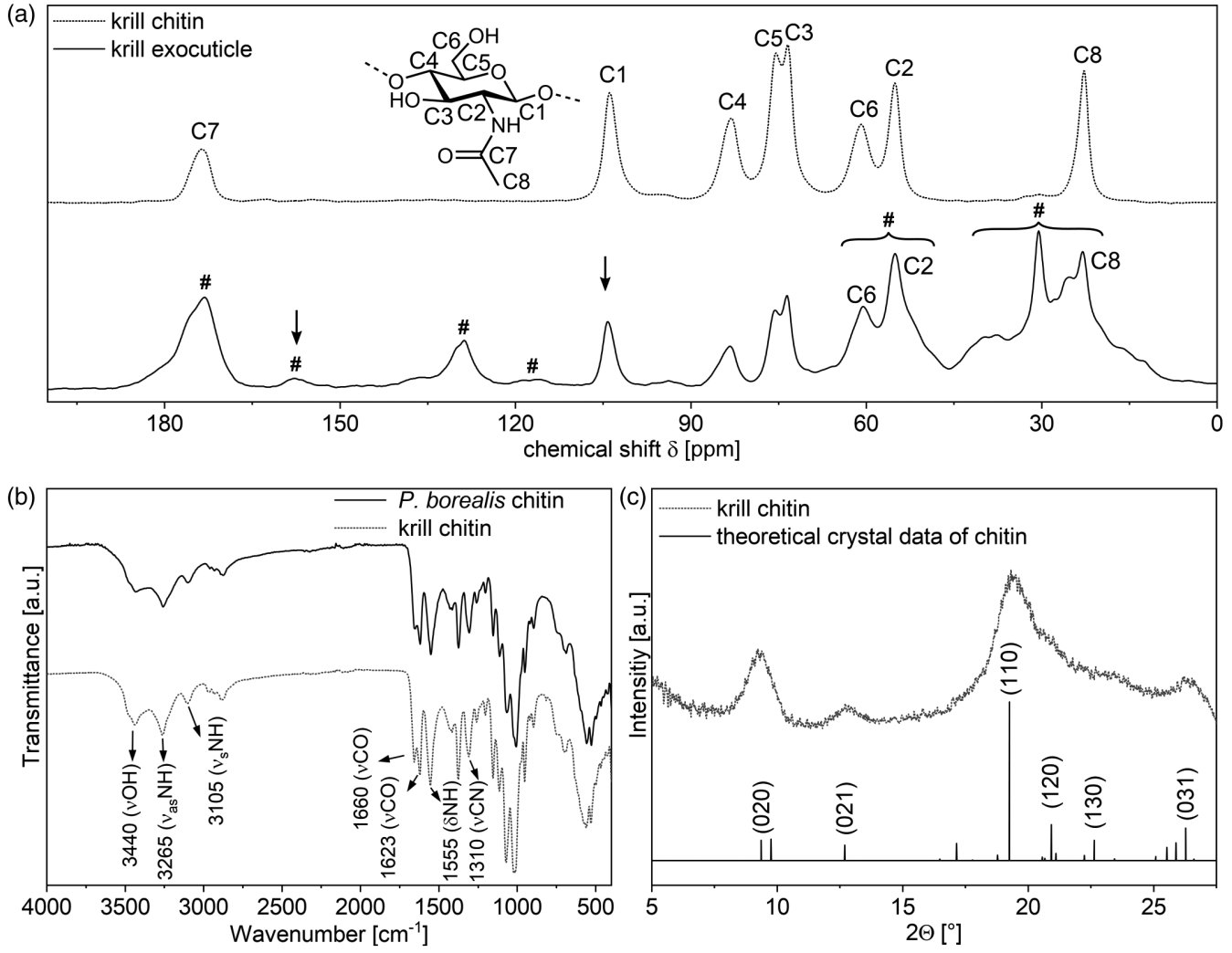

FIGURE 3 Spectroscopic characterization of the purified krill chitin compared to *P. borealis* chitin and the raw biomass: (a) shows the ^{1}H-^{13}C CP MAS NMR of the krill exocuticles and the purified chitin. The hash (#) marks the chemical shifts of the proteins, which can overlap with the chitin signals. The arrows label the peaks used for the protein quantification; (b) shows the FT-IR spectra of the *P. borealis* chitin and the krill chitin; (c) illustrates the XRD diffractogram of the krill chitin and the generated diffractogram from crystal data of α-chitin.

applied to assess qualitative information on chitin such as the polymorphic forms, presence of impurities and the degree of acetylation (DA).[31–33] The spectra of krill chitin reveals peaks at 173.1 (C7), 104.3 (C1), 83.2 (C4), 75.8 (C5), 73.5 (C3), 60.9 (C6), 55.2 (C2), and 22.9 (C8) ppm (see Figure 3a) representing the common peaks for

purified chitin.[34] Impurities such as proteins lead to additional peaks in this spectral region that overlap with the peaks of chitin (see the spectrum of native krill exocuticle in Figure 3a). ^{1}H-^{13}C CP MAS NMR can also be applied to gain quantitative information about the raw material via a non-destructive way. Cross-polarization (CP) leads to a strong signal enhancement of diluted nuclei. It is not leading to an equal excitation of all ^{13}C nuclei in the sample at the same time, as the strength of the polarization transfer depends on chemical environment, distances and dynamics of involved nuclei.[35] A peak integration is thus usually not leading to quantitative results, the errors arising from application of CP might compensate each other to a reasonable extent if proper CP conditions are used. Such conditions were identified by Ottey et al.[36] and give reliable results for the determination of the DA that are in the range of other approaches. Thus, the method gained wide acceptance for the quantitative investigation of chitin within the errors of a chemical analysis. The same conditions are herein used for the quantitative investigation of the protein content of native krill exocuticles and compared to an amino acid analysis. The quantification is based on integration of the C1 signal at 104 ppm of the chitin and the ε-/ξ-carbon peaks at 157 ppm of arginine and tyrosine (see Figure 3a—black arrows). Those are free from signals of lipids and other amino acids.[37,38] The protein-to-chitin ratio (Section 4.3.6 Equation 6) of the krill exocuticle is calculated from the integral quotient by using mole fractions of arginine and tyrosine in the total protein content. These values could be calculated considering published protein compositions determined with the whole krill biomass.[27,39] The protein content of 35 ± 3% by weight is determined via multiplying the protein-to-chitin ratio by the chitin content (19.8%) via ADF-ADL (see Section 4.3.3). Compared to the protein content derived from amino acid analysis of the exocuticle (Section 4.3.5, 39.8%) the proposed ss-NMR methods seem to give adequate results.

Figure 3a indicates the effectiveness of the purification steps. The DA of the purified chitin is determined to be 88%.[36,40] In comparison, the DA of crustacean-derived α-chitin is usually between 80% and 98%.[31,41–43]

FT-IR of krill-derived chitin give prominent bands at wavenumbers of 1310 cm^{-1} (CN stretching, amide III), 1555 cm^{-1} (NH bending, amide II), 1623 and 1660 cm^{-1} (CO stretching, amide I), 3105 cm^{-1} (NH symmetric stretching), 3265 cm^{-1} (NH asymmetric stretching), and 3440 cm^{-1} (OH stretching) (see Figure 3b, see commercial chitin from *P. borealis* for reference).[44,45] The two separate bands at 1623 and 1660 cm^{-1} indicate the presence of the polymorphic structure α-chitin. The polymorphism is correlated to the occurrence of the intermolecular hydrogen band —CO•••HN— and —CO•••$HOCH_2$.[46]

Powder x-ray diffraction shows broad reflections at 2θ values of 9° (0 2 0), 13° (0 2 1), 20° (1 1 0), 21° (1 2 0), 23° (1 3 0), and 26° (0 3 1) indicating the occurrence of the lattice planes (0 2 0), (0 2 1), (1 1 0), (1 2 0), (1 3 0), and (0 3 1) of α-chitin (see Figure 3c).[47–49] The crystallinity index (CI_{110}) of the chitin was calculated to be 76%.[49,50] The XRD results revealed that the CI of chitin isolated from krill exocuticles is located within the scope of the CI values of crustacean-derived α-chitin (64%–93%), whereby the variation is due to the species and purification methods.[41,47,51–54]

2.2 | Deacetylation of chitin

2.2.1 | Evaluation of the deacetylation process

Chitin can be transformed into its more applicable derivative chitosan. Chitosan is less acetylated than chitin, and therefore, shows different solubility properties. For the removal of the acetyl groups different literature-based methods were investigated regarding their chitosan content, and its deacetylation degree. The biomass loss of the procedures DAFR and DAY was 17.4% and 23.3%, respectively.

After deacetylation, the biomass was purified two times by solubilization and precipitation. The obtained chitosans were analyzed.

2.2.2 | Chitosan analytics

The produced chitosan was evaluated using various analytical methods. The data achieved is shown in Table 4.

With the deacetylation methods DAY and DAFR chitosan yields of 60% and 62%, respectively, were achieved after the second solubilization and precipitation step. The ash content of the chitosan was determined to 1.5% and 1.6% for DAY and DAFR, respectively. Most chitosan contains less than 1% ash after deacetylation, like Yuan et al.[14] with 0.4% ash in chitosan produced from Antarctic krill exocuticles. The deacetylation degree (DDA) within our study was measured via Elemental Analysis and NMR. These methods resulted in different DDA, while NMR showed the higher ones (DAY: 88%, DAFR: 74%) than EA. ^{1}H NMR spectroscopy is said to be the most accurate method for the determination of the deacetylation degree of chitosan, as it is less sensitive to impurities than elemental analysis.[55] According to Younes et al.[56] deacetylation degree of chitosan obtained from

TABLE 4 Evaluation of the produced chitosans after deacetylation.

	DAY	DAFR
Yield dry biomass (%)	88	85
Yield chitin (%)	60	62
Ash (%)	1.5	1.6
M_p (kDa)	31.0	46.0
M_n (kDa)	13.1	16.8
M_w (kDa)	101.7	126.3
PDI	7.8	7.5
%C	42.3	40.7
%H	7.3	7.1
%N	7.7	7.1
DDA (%, EA)	78	66
DDA (%, NMR)	88	74
Film formation	All films were transparent and homogeneous	

Note: Yield dry biomass is based on the initial weight of the intermediate material obtained from deproteinization used in the deacetylation, yield chitin describes the yield regarding the specific chitin content in the intermediate material.

shrimp waste under air atmosphere, like herein, was around 90%–93%. A similar DDA was determined by NMR using the DAY method. Gagnieu et al.[57] determined a DDA of 85% for the chitosan obtained. The DDAs determined here by this method (DAFR) were significantly lower at 66% (EA) and 74% (NMR). Yuan et al.[14] reported a DDA of only 75% for chitosan produced from Antarctic krill chitin.

Film forming assay was done by solubilization of the obtained chitosans in acetic acid, pouring the solutions into a Petri dish and drying them until they formed a film. The formed films were both transparent and homogenous.

DAY and DAFR chitosans achieved a comparable molecular weight of 101.7 and 126.3 kDa, respectively. The determined molecular weights are relatively low compared to values published by other authors applying viscosimetry for the determination. For example, Yuan et al.[14] determined 1110 kDa for a chitosan from Antarctic krill. In some cases, however, lower polymer lengths were determined, such as 157 kDa by Wu et al.[58] The concentration of sodium hydroxide solution, temperature, and incubation time can generally have a negative influence on the molecular weight. $NaBH_4$ that was used in these methods should technically act as a reducing agent and accelerate deacetylation as well as reduce oxidation of the chitosan.[7] Long storage of the exocuticles and chitosans can also have a negative impact on the molecular weight.[59] With a low molecular weight and a high DDA between 70% and 90% the chitosan produced in this study is especially suitable for applications in wound healing, food preservatives and waste water treatment because of its high solubility, low viscosity, antimicrobial, and adsorbing properties.[60]

Based on the biomass of origin, the krill exocuticle, a chitosan yield of 10.7% (DAY) and 11.1% (DAFR) was obtained. The reported yield is corrected for impurities and indicates the actual chitosan content. In light of recent studies, Antarctic krill exocuticles are a competitive chitosan source. Saravanan et al.[61] recently obtained comparable yield (16%) and DDA (72%) from related shrimp exocuticles, although impurities were not considered in this work.

3 | CONCLUSION

By this research, purified chitin and thus chitosan was obtained from Antarctic krill exocuticles.

Degreasing with ethanol as solvent was identified as suitable first step of the purification procedure exhibiting the highest yields of krill oil and phospholipids. On one hand, this increases the purification degree of the chitin, on the other hand it offers the opportunity to initialize a new value chain.

Demineralization was performed with an efficiency of about 93% using citric acid which is similar to the efficiency achieved with corrosive hydrochloric acid. The high content of citrate and metal ions like Mg^{2+} and Ca^{2+} make the effluent suitable as supplement or fertilizer. A deproteinization efficiency of 99.9% was achieved within this research work. 79%–100% of the chitin was recovered from the chitin applied at the beginning of the process within the biomass confirming the suitability of the given approaches. To increase the sustainability of this process step and to gain a protein hydrolysate with potential for further use, it would be beneficial to screen for proteases enabling the deproteinization at mild conditions.

Our investigations revealed a chitin with a purification degree of 93% and a DDA of 88% determined by ss-NMR. Besides, our investigations confirmed that the protein content determination in the raw material via ss-NMR is valid.

The chitin conversion was conducted at two different (harsh) conditions leading to chitosans with DDAs of 74% and 88%, low ash contents and low molecular weights but considerably high PDIs (~7.5). It could be expected that chitosan isolated from fresh biomass reveals a narrower molecular weight range. As it is the case for deproteinization, a biotechnological chitin conversion process would be of benefit especially considering the drastic conditions.

4 | EXPERIMENTAL SECTION

All weight specifications are related to the dry matter (93.5%). The experiments were performed as triplicates with exception of the deacetylation trials, which were performed once.

4.1 | Materials

The krill exocuticles were derived from frozen krill, which was caught in the Antarctic Atlantic in the fishery zone 48.3 (South Georgia). The krill was thawed, minced and mixed with water in a ratio 1:1. Proteolytic enzymes were added to the mixture and the mixture was stirred gently for 30 min. Finally, the exocuticles were removed by decantation and dried. The exocuticles were crushed into a particle size of 0.5 mm.

The commercial chitin purified from *P. borealis* was purchased from Chitinor AS (Norway) and applied for analytical comparison purposes.

In general, the reagents used were of analytical grade or of technical grade (n-hexane, acetone, and ethanol). If not stated otherwise, demineralized water was applied.

4.2 | Methods

4.2.1 | Degreasing evaluation

Organic solvents have been used to degrease coarse krill exocuticles. Therefore, the following solvents were chosen: Ethanol, since it is known as a conventional solvent for extracting krill oil[14] and acetone, as it can separate neutral lipids.[62] A 1:1 (v/v) acetone/ethanol-mixture was also applied according to the one-step process of Gigliotti et al.[20]

First, 20 g of the krill exocuticles were suspended in 240 mL of solvents (solid–liquid ratio of 1:12 [w/v]) in 500 mL bottles. The extraction was carried out at RT overnight under continuous stirring (250 rpm), followed by 30 min centrifugation at 4696 *g* at RT to separate solid and liquid. After decanting, the krill oil was recovered from the supernatant using a rotary vacuum evaporator at 60°C and 280 mbar (ethanol), 750 mbar (acetone), and 600 mbar (acetone/ethanol), respectively. Finally, the processed krill exocuticles were dried at RT under the fume hood for about 4 days. Residual solvent content was measured and considered to generate the mass balance.

For comparison purposes, oil was also extracted from the exocuticles using Soxhlet extraction.[62] This extraction was carried out with 7 g krill exocuticles and 150 mL of n-hexane in a standard Soxhlet apparatus for 5 h, followed by recovery via rotary vacuum evaporator at 50°C and 400 mbar.

In addition, the weight of krill oil obtained with the different solvents was recorded to determine extraction efficiency according to the following equation:

$$\text{Yield}\,(\%) = \frac{m_1\,(\text{g})}{m_2\,(\text{g})} \cdot 100 \quad (1)$$

where, m_1 (g) is the dry weight of extracted krill oil, m_2 (g) is the dry weight of initial krill exocuticles.

For large scale-approaches degreasing was performed with 350 g dry weight of krill exocuticles in the same solid/solvent ratio of 1:12 in ethanol in a 5 L bottle at 130 rpm. Filtration (filter paper 4–7 μm) was used for separating the solid from the solvent. Residual biomass was dried at 105°C.

4.2.2 | Demineralization evaluation

For the demineralization investigations, ethanol-degreased krill exocuticles, produced regarding the protocol in Section 4.2.1, were applied. Demineralization of the krill exocuticles should be conducted to remove the carbonate salts of metal ions. Demineralization evaluation was done by mixing 2.5 g of degreased krill exocuticles with 1 M of the certain acid at a 1:10 (w/v) ratio in a shake flask (100 mL) at 150 rpm and RT overnight. As demineralization agents, citric, formic, hydrochloric, and malic acid were selected. An incubation with water was carried out as benchmark. The samples were filtered using a glass filter frit (pore size: 40–100 μm) and washed with water to neutral pH. The washed residual biomass was dried at 105°C overnight. Demineralization efficiency was calculated by measuring ash content before and after demineralization with the following equation:

$$\text{Demineralization efficiency}\,(\%) = 100 - \frac{m_1(\text{g})}{m_2\,(\text{g})} \cdot 100 \quad (2)$$

With m_1 (g) being the measured ash content after demineralization and m_2 (g) being the measured initial ash content of the krill exocuticles before demineralization process.

Citric acid-based demineralization was identified to be the most suitable process to remove the minerals. To assess the further application of the supernatant, it was lyophilized and subjected to compositional analysis according to SS-EN ISO 17294-2:2016. Demineralization in larger scale was conducted with the obtained biomass of the large-scale degreasing approach with 1 M citric acid (S/L 1:10) in a 5 L bottle.

4.2.3 | Deproteinization optimization

Deproteinization was performed under various conditions using the citric acid-demineralized krill exocuticles. Different deproteinization conditions were analyzed in terms of their deproteinization efficiencies for the krill exocuticles. Therefore, the Radley's Tornado™ system (IS6, UK) with continuous agitation was used with the following conditions: (1) 2 M NaOH, 90°C, 2.5 h, (2) 1 M NaOH, 80°C, 3 h, (3) 2 M NaOH, 70°C, 4 h, and (4) 2 M NaOH, RT, 24 h.[25,28,30,63,64] A solid:liquid-ratio of 1:10 (w/v) was maintained for all approaches. Afterwards, the resulting chitin was filtrated in the heat and washed with warm water until reaching pH neutrality. The residual biomass was dried at 60°C until dryness. To evaluate the success of the process, ash, protein, and chitin content were measured. Deproteinization efficiency was calculated with the following equation:

$$\text{Deproteinization efficiency}(\%) = 100 - \frac{\left(\left(\frac{w_1}{m_1}\cdot 100\right) - \left(\frac{w_2 \cdot w_3}{100}\right)\right)\cdot 6.25}{w_4}\cdot 100 \quad (3)$$

With m_1 (%) is the dry mass of the deproteinized krill exocuticles, w_1 (%) is the nitrogen content in moist mass measured by elemental analysis, w_2 (%) is the chitin content in the deproteinized krill exocuticles measured by ADF-ADL analysis, w_3 (%) is the nitrogen content of krill chitin, assuming an acetylation degree of 91% (determined via solid-state-NMR) resulting in 7.1% (w/w) nitrogen, and w_4 (%) is the total protein content (40.3%) in the original krill exocuticles measured by elemental analysis (see Section 4.3.2).

Chitin recovery is calculated based on Equation (4). With w_2 (%) as the chitin content after deproteinization measured by ADF-ADL, and $w_{\text{exocuticle}}$ (%) as the chitin content in the Antarctic krill exocuticle, which was determined with 19.8%.

$$\text{Chitin recovery}\ (\%) = \frac{w_2}{w_{\text{exocuticle}}}\cdot 100 \quad (4)$$

Larger amounts of deproteinized biomass were prepared on two occasions in a 1 L double jacketed glass reactor with segment helical stirrer at 150 rpm instead of in the Tornado system.

4.2.4 | Purification and deacetylation

Deacetylation of chitin is performed to convert the chitin into chitosan. The investigations were performed as single approach. The two following methods were investigated:

1. Deacetylation according to Younes et al.[56] (DAY)

   In the present study, a mixture of 50% (w/v) NaOH and 5 g $NaBH_4$ was used for the deacetylation of 5 g (S/L 1:20 w/v) chitin at 120°C for 3 h. Therefore, 15 g of chitin were treated according to these specifications except for lowering NaOH concentration to 40% (w/v) and adding 15 g $NaBH_4$. The approach was conducted in a 500 mL three-neck PFA round bottom flask heated by an oil bath under reflux.

2. Deacetylation according to Gagnieu et al.[57] (DAFR)

   This patent describes the deacetylation of chitin in a hot aqueous alkaline medium containing ethanol and a metal hydroxide. Therefore, a mixture of 75 g of water, 150 mL of ethanol, 0.23 g of $NaBH_4$, and 75 g of NaOH pellets was made. Then the mixture was preheated to 90°C in a 500 mL three-neck PFA round bottom flask heated by an oil bath before adding 20 g of chitin for 4 h under stirring.

Temperatures varied by ±3°C. All experiments were conducted under stirring (120 rpm). After each deacetylation, the mixture of chitosan and liquid was filtrated (filter paper, pore size: 40 µm) for separation, and the solid was washed until the wash water reached neutral pH. The obtained chitosan was dried at 60°C overnight. Five grams of the residual biomass was dissolved in 1% (v/v) acetic acid in a solid/liquid ratio of 1:50 w/v for 24–72 h for purification. Then, insoluble particles were removed by filtration (glass filter, pore 16–40 µm, 125 mL). The dissolved chitosan was then precipitated by titration with 40% (w/v) NaOH to a pH around 8. Subsequently, the residual solid was washed with a glass filter (pore 16–40 µm, 1000 mL) with water until the wash water reached a neutral pH. For a thorough purification, the solubilization–precipitation procedure was repeated, precipitating this time at pH 10. As the final step, the chitosan was dried at 30°C in a High-vacuum drying oven at 70 mbar and subjected to ash and DDA analysis.

4.3 | Analysis

The moisture and ash contents were determined at 105 and 550°C according to DIN EN 15935:2009 and 12880:2000.

4.3.1 | Loss of biomass

The amount that was lost during each step of the chitin extraction and chitosan production was defined as described below:

$$\text{Biomass loss}\,(\%) = 100 - \frac{m_1\,(\text{g})}{m_2\,(\text{g})} \cdot 100 \qquad (5)$$

where, m_1 (g) is the mass of the product after the process and m_2 (g) the mass of the substrate before the process.

4.3.2 | Elemental analysis

The content of the carbon, hydrogen, and nitrogen in the samples was measured via elemental analysis with a Euro EA HEKAtech. For this procedure, 10 mg of sample were weighed in a tin capsule and degraded at 1100°C under oxygen flux. The resulting gases were converted with a reactive oxidation catalyst and separated with a gas chromatography column. Detection was conducted with a thermal conductivity sensor.

4.3.3 | Chitin content

The chitin content of the raw material, the demineralized and deproteinized biomass was measured as acid detergent fiber (ADF). The value of the acid detergent lignin (ADL) was measured and deducted from ADF to consider catechol-similar compounds according to Hahn et al.[65] In general, the ADL value is to be negligible for krill-based samples.

4.3.4 | Protein content via elemental analysis

Protein content was determined via the nitrogen content in the biomass subtracted with the nitrogen incorporated in the chitin according to Hahn et al.[66] For evaluation purposes, protein content was also determined after each treatment.

4.3.5 | Protein content via amino acid analysis

32.644 mg was accurately weighed into a hydrolysis tube, overlaid with 1 mL of a performic acid solution and oxidized for ~24 h at 0–5°C. Excess oxidation reagent was destroyed by addition of sodium disulfite. To each of the oxidized samples, 5 mL of 6 N hydrochloric acid was added and hydrolyzed for 24 h at 110°C (microapparatus with reflux condenser). The sample was then dried at 40°C in the SpeedVac for 14 h under vacuum. The dried sample was taken up in 5 mL sample dilution buffer (Na-acetate buffer, pH 2.2), suspended solids were centrifuged, and after further dilutions, if necessary, fed to the chromatographic measurement. A polymeric cation exchange column was used to separate amino acids by HPLC chromatography (particle size: 4 µm; column dimensions: 125 × 4 mm ID) with a sample volume of 20 µL. Separated amino acids were detected by post-column Ninhydrin derivatization at 125°C and photometric measurement at 570 nm.

4.3.6 | Solid-state-NMR

^{1}H-^{13}C CP MAS NMR experiments were performed on an Avance III 400WB spectrometer at a resonance frequency of 100.6 MHz, a spin rate of 8 kHz and by applying a contact time of 1 ms as conducted by Ottey et al.[36] A delay of 5 s between the scans was applied.

The protein content was quantified with the protein-to-chitin ratio by the following equation

$$\text{Protein content}\,(\%) = \frac{I_{\text{AA}}}{I_{\text{C1}}} \cdot \frac{n_{\text{C1}}}{n_{\text{AA}}} \cdot \frac{1}{X_{\text{AA}}} \cdot w_{\text{Chitin}} \qquad (6)$$

where, I_{C1} is the integral of the C1 carbon of chitin at 104 ppm, I_{AA} is the integral of the ε-/ξ-carbon of the amino acids tyrosine and arginine at 157 ppm, n_{C1} is the number of chitin nuclei at 104 ppm, n_{AA} is the number of amino acid nuclei at 157 ppm, w_{Chitin} is the chitin content determined by the already mentioned ADF-ADL method. X_{AA} (*E. superba*, whole krill: Tyr: 0.0359, Arg: 0.0506)[27,39] is the mole fraction of tyrosine and arginine calculated with the amino acid profile published by other authors. The mole fraction X_{AA} was calculated by using the formula

$$X_{\text{AA}} = \left(\frac{w_{\text{Arg}}}{M_{\text{Arg}}} + \frac{w_{\text{Tyr}}}{M_{\text{Tyr}}} \right) \cdot \overline{M} \qquad (7)$$

where, w_{Arg} is the mass fraction of the arginine, w_{Tyr} is the mass fractions of the tyrosine, M_{Arg} is the residual molar mass of arginine (156.19 g/mol), M_{Tyr} is the residual molecular mass of tyrosine (163.18 g/mol), and $\overline{M}$ is the average residual molecular mass of the amino acids.

The residual molecular mass was used since water is split off during the polycondensation reaction of the amino acids.

The DA was calculated from the integral of the methyl carbon C8 divided by the integrals of the ring carbons C1–C6[36,40]

$$\mathrm{DA}\,(\%) = \frac{I_{C8}}{(I_{C1}+I_{C2}+I_{C3}+I_{C4}+I_{C5}+I_{C6})\cdot\frac{1}{6}}\cdot 100 \quad (8)$$

4.3.7 | ^{1}H-NMR

5-10 mg of the chitosan sample was solubilized in 700 µL of 2 % (w/w) deuterium chloride (DCl) in deuterium oxide (D_2O) for 1 h at 70 °C. The samples were then analyzed by a Bruker Avance 500 NMR spectrometer at 500 MHz. For determination of the DDA, the ratio of the relative mean integral of the protons of the acetyl group I_{H8} (1.95 ppm) and the mean integral of the protons of the monosaccharide ring $I_{H2\text{-}H6}$ (2.7-4.4 ppm) were calculated.

4.3.8 | AT-IR

The FT-IR spectra were recorded on a "Vertex70" spectrometer (Bruker) using a diamond ATR crystal. The FT-IR measurements were executed in the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1} and a measuring time of 60 scans (>25 s).

4.3.9 | XRD

The chitin was put into a capillary (ø = 7 mm). The XRD measurements were performed with a Bruker AXS Nanostar. It has a copper anode ($\lambda = 1.5418$ Å) and is equipped with a Histar 2D detector, which provides two-dimensional roentgen scatter images. To obtain the scattering intensity as a function of the scattering angle, the obtained 2D images were integrated over one direction in space. The scattering angle was calibrated by using silver behenate. The measurement was performed at 25°C.

The crystallinity index (CI) was determined by using the equation

$$\mathrm{CI}_{110}\,(\%) = \frac{I_{110}-I_{\mathrm{am}}}{I_{110}}\cdot 100 \quad (9)$$

where, I_{110} is the maximum intensity of the (1 1 0) lattice diffraction at $2\theta = 19°$ and I_{am} is the intensity of the amorphous diffraction at $2\theta = 12.6°$.[49,50]

4.3.10 | Size exclusion chromatography

For molecular weight analysis a HPLC System (Bischoff GmbH, Leonberg, Germany) equipped with a Catphil-P-400 × 8 mm column (Applichrom, Oranienburg, Germany) was used. A solution of 0.075 M $NaNO_3$ with 0.3% formic acid was established as the mobile phase. Around 5 mg/mL sample were dissolved overnight in the mobile phase and filtered through a 0.22 µm nylonfilter to remove particles. The sample was then measured with an injection volume of 50 µL, 0.8 mL/min flow and a runtime of 30 min.

4.3.11 | Film formation of chitosan

In order to determine the film-forming ability, 0.1 g of chitosan were dissolved in 10 mL 1% (v/v) acetic acid and poured into a Petri dish with a diameter of 150 mm. With open lid, the chitosan solution was dried at RT for 3 days. The film formation was evaluated visually.

4.3.12 | Analysis of the degreasing supernatant

Lipid class analyzes were performed by a HPLC system (PerkinElmer, Waltham, USA) equipped with an ESA Corona® Plus Charged Aerosol Detector (ESA Biosciences, Inc., Chelmsford, USA). The samples were separated on a LiChrosphere® 100, 5 µm diol column, 4 × 125 mm (Merck KGaA, Darmstadt, Germany). A ternary gradient consisting of solvent A = isooctane, B = acetone/dichlormetane (1:2), and C = 2-propanol/methanol/acetic acid-ethanolamine-water (7.5 mM ethanolamine and 7.5 mM acetic acid) (85:7.5:7.5) was used with the following profile: at 0 min, 100:0:0 (%A/%B/%C); at 1 min, 90:10:0; at 8 min 70:30:0; at 11 min 40:50:10; at 13 min 39:0:61; at 26.3 min 40:0:60; at 28.4 min 0:100:0; at 30.9 min 100:0:0. The lipid components were identified by comparison to the retention time of commercial standards.

AUTHOR CONTRIBUTIONS

Thomas Hahn: Conceptualization (lead); funding acquisition (lead); methodology (lead); supervision (lead); writing – original draft (lead). **Jeannine Egger:** Investigation (lead); methodology (supporting); writing – original draft (supporting). **Simon Krake:** Data curation (supporting); investigation (supporting); writing – original draft (supporting). **Michael Dyballa:** Data curation (supporting); investigation (supporting); resources (supporting); writing – review and editing (supporting). **Linus Stegbauer:** Conceptualization (supporting); investigation (supporting); writing – review and editing (supporting). **Nils von Seggern:** Data curation (supporting); investigation (supporting); writing – review and editing (supporting). **Inge Bruheim:** Funding acquisition (lead); project

administration (supporting); supervision (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Susanne Zibek:** Funding acquisition (lead); project administration (supporting); supervision (equal); writing – original draft (supporting); writing – review and editing (supporting).

ACKNOWLEDGMENTS

The authors would like to thank Padma Priya Ravi for their valuable comments during generation of the manuscript. Additionally, we would like to thank Jessica Bauhof for the XRD measurements. Open Access funding enabled and organized by Projekt DEAL.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: T. Hahn, J. Egger, S. Krake, M. Dyballa, L. Stegbauer, N. von Seggern, I. Bruheim, S. Zibek, *J. Appl. Polym. Sci.* **2024**, *141*(2), e54789. https://doi.org/10.1002/app.54789