Protein fractionation, bioactivity analyses and large scale processing of mussel meat

Deliverable for project KB34-3A-1 'Diagnostics for biorefinery of low trophic marine resources to animal health application'

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This research project has been carried out by Wageningen Food & Biobased Research commissioned by the Dutch Ministry of Agriculture, Nature and Food Quality.

Wageningen Food & Biobased Research
Wageningen, December 2022

Confidential until December 2025

Report 2380
DOI 10.18174/584145

WAGENINGEN
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WFBR Project number: 6224092600
KB Project number: KB34-3A-1
Version: final
Reviewed and approved by: Jan Jetten
Commissioned and funded by: the Dutch Ministry of Agriculture, Nature and Food Quality

Confidential until: December 2025

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1 Preface

This report is a 2022 deliverable for project KB34-3A-1 'Diagnostics for biorefinery of low trophic marine resources to animal health application'. It describes the work done in 2022 for the process development, and also includes the work for the deliverable on the bioactivity assay results.
In short, the activities in 2022 include:

- Development of pH-swing method for protein separation
- Further analyses of the bioactivity in different fractions in the developed process
- Large scale production of material following the optimised route for a feed trial
- Production of material for the work performed at WLR and WFSR

2 Introduction

2.1 Goal

The process development in 2022 has focussed on the production of mussel material on a larger scale. The material is required for both *in vitro* and *in vivo* assessments, performed by the Wageningen Livestock Research. Also, for each processing step, material is required for the study on resource and process hazards at Wageningen Food Safety Research.
The effect of the larger scale processing on the process yield and the bioactive properties of the material was studied. The conditions for processing were chosen based on the findings of the study of 2021 (Optimisation of biorefinery process for mussel protein fractions, report 2252).
In addition, an alternative method for the production of bioactive fractions was studied, using pH swing. The idea behind this is that sarcoplasmic (blood) proteins, that commonly solubilise at pH 6, have different bioactive properties as compared to the myofibrillar proteins that dissolve at pH 10. Separate fraction were therefore be assessed on bioactivity. Filtration was be attempted to create separate fractions with different molecular weight.

2.2 Background

Low trophic marine biomass will likely play an important role in the development of circular and climate positive food and non-food production systems. This biomass, such as seaweed and shellfish, can be cultivated for direct use as food and feed. For some types of biomass, health promoting (bioactive) properties may be present, aside from nutritional value. Using biorefinery, it would become possible to produce several fractions of the biomass, each with its application, hereby ensuring higher resource efficiency and maximum valorisation.

The effect of feeding mussel meat to cultured sole (*Solea solea*) has been monitored in the previous year. It appears that the inclusion of mussels in the fish feed reduces the occurrence of anaemia in sole. It is hypothesized that the anti-oxidant activity that is found in the mussels in an *in vitro* assay is, at least partly, responsible for this reduction in anaemia. The specific components that give the mussel the anti-oxidant activity are unknown. It would be possible that specific peptide, known to possess such properties, are present in the matrix and cause the anti-oxidant activity.

3 Material and methods

3.1 Material

For these experiments, mussels from the brand Fish Tales were used (Figure 1). These mussels were aqua farmed at the Oosterschelde and are also known under the name 'hanging culture'. This batch was harvested in January of 2022.

Figure 1 Mussels as starting material

3.2 Mussel small scale processing

At a laboratory setting mussels were processed in order to identify key processing parameters related to mass balances, protein contents and the bioactivity of specific fractions.

3.2.1 Pretreatment

The mussels were blanched for 30 seconds at 98°C. After blanching, the shells were separated manually from the mussel meat. The mussel meat was disrupted with a blender (Vitamix 5200) for 30 seconds.

3.2.2 Mussel fractionation

After blending, the pH of the material was measured and adjusted to pH 6 in order to standardize and solubilize the sarcoplasmic protein. Subsequently, the material was centrifuged (16,000×g, 20 min, 4 °C) to recover a liquid fraction and pellet. The liquid fraction (SN 1) was further processed. The pellet (pellet 1) was washed with water and centrifuged. Both the pellet (pellet 2) and supernatant (SN 2) were set aside (Figure 2).

In order to increase the solubility of the particles in the liquid fraction (SN 1), the pH was adjusted to pH 10. After that, the fraction was centrifuged (16,000×g, 20 min, 4 °C). The supernatant (SN 3) was further processed, the pellet (pellet 3) was not further processed.

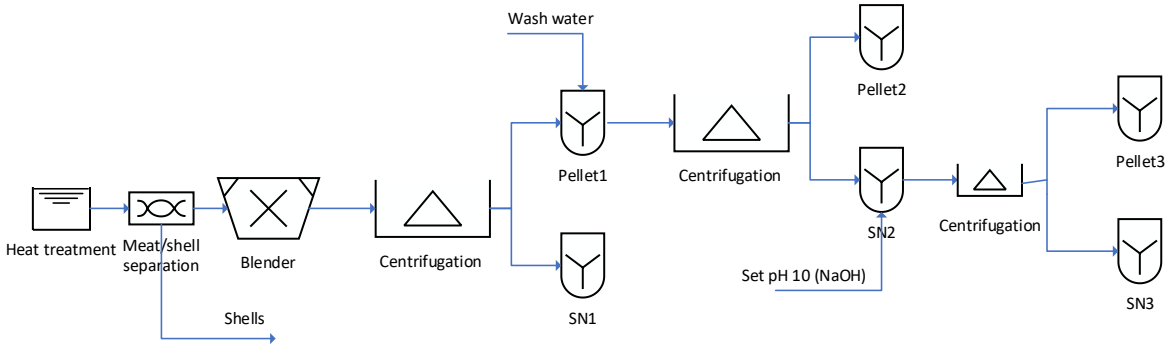

Figure 2 ***Process for mussel fractionation***

3.2.3 Separation of liquid fraction

The supernatant fractions SN1 and SN2 combined (Figure 3) were analysed on molecular size. Therefore a size exclusion chromatographic column (Superdex 200) with an eluens of 20 mM Tris at pH 6 with 0.2 M NaCl was used. Based on the particle size distribution two filter sizes were selected to separate high, medium and low molecular weight proteins. The filters used were 300 kDa and 5 kDa Greiner centrifugal filter tubes of 50 mL. Tubes were centrifuged in a Sorval F14 rotor at 33,000 ×g for 40 minutes at 20°C. After separation, the pH of the fractions was adjusted to pH 6. All the fractions were freeze-dried.

3.2.4 pH swing fractionation

Protein from legumes is often extracted by the use of a pH swing. A similar protocol was used for the extraction of protein from the pellet after washing (pellet 2), as displayed in Figure 3. Protein from the washed pellet (pellet 2) can be extracted using a pH swing. The protein from the pellet is solubilized at around pH 10, followed by a precipitation at pH 4. The material was centrifuged (16,000×g, 20 min, 4°C) to recover protein in the precipitate (pellet 4). The supernatant (SN 4) was set aside.

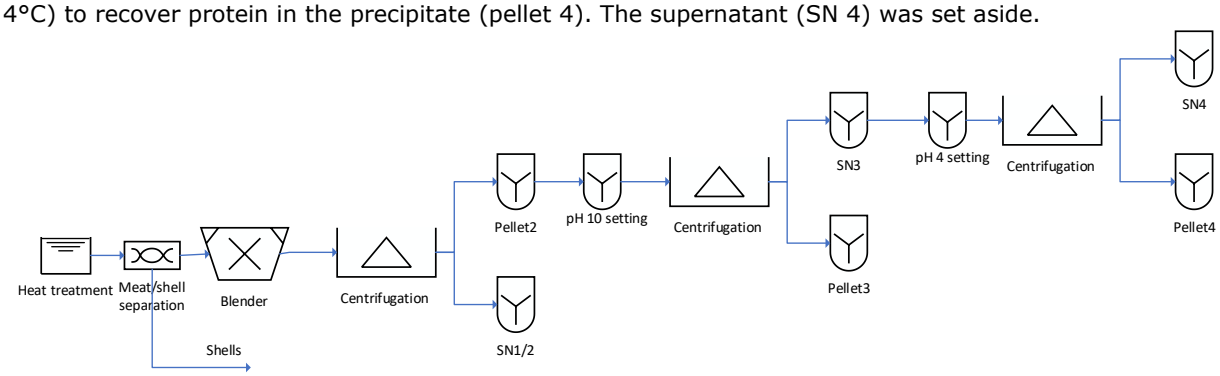

Figure 3 ***Process flow sheet for fractionation of protein by pH swing***

Another pH swing process was used in order to extract protein from the mussel meat. This process was a direct pH swing and is displayed in Figure 4. The mussels were blanched, separated from shell and blended. After blending, water was added to the slurry. The pH of the slurry was adjusted to 10 and the mixture was stirred for 1 hour. After that, the mixture was centrifuged (16,000×g, 20 min, 4°C) and the pellet was set aside. The pH of the supernatant was adjusted to 4 and was stirred for 1 hour. After that, it was centrifuged (16,000×g, 20 min, 4°C) and the precipitate and liquid were separated.

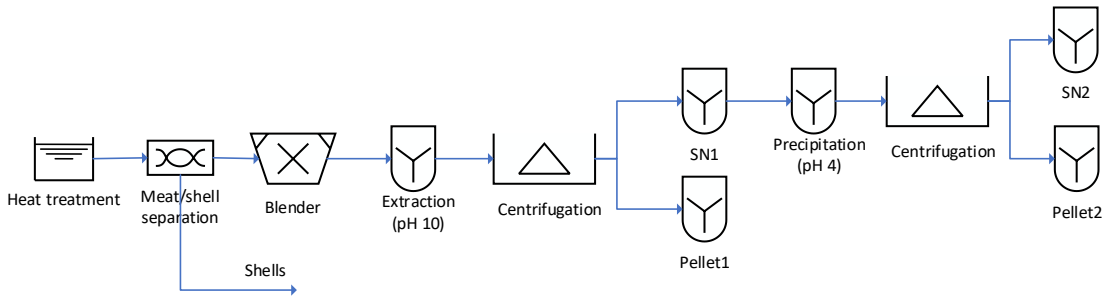

Figure 4 ***Process flow sheet of direct pH swing***

3.3 Large scale mussel processing

3.3.1 Material

For the large scale extraction of the bioactive fraction, Jumbo sized 'Zeeuwse' mussels from Driessen food were used (batch L49624). The process consisted of the following steps:

- Blanching
- Pealing by hand
- Grinding
- pH setting
- Solid/liquid separation

3.3.2 Equipment

Blanching of the mussels was performed in an ANBO blanching unit, grinding of the mussel meat was performed with a colloid mill (Figure 5). For the mixing and pH setting two 75 L jacketed vessels with a double bladed propellor stirrer were used. Solid liquid separation was performed in a Sorval Lynx fixed angle centrifuge with 6x 1 L buckets.

Figure 5 ***Blanching unit (L) and colloid mill (R) used for large scale processing of mussels***

3.3.3 Method

For the large scale extraction, the mussels were blanched in a water bath for 30 seconds and transferred into an ice bath for 5 min. After that, the shells were removed and the mussel meat was collected. Unopened mussels and those with damaged shells were discarded.

The mussel meat was transferred into the colloid mill to grind the meat. After that, the mussel slurry was placed inside the stirred tanks and the pH was adjusted to pH 6 with 1M NaOH. The slurry was stirred for 30 minutes at room temperature. Subsequently, the slurry was centrifuged at 17,500 ×g

for 20 minutes. The supernatant and pellet were separated and both freeze-dried. The protein content was measured with Kjeldahl using a nitrogen conversion factor 6.25.

3.4 Bioactivity analyses

After freeze drying, the antioxidant capacity of the samples was analysed using the DPPH protocol. The DPPH protocol can be found in deliverable "Further development of antioxidant assay for screening of specific bioactivity of samples". The anti-oxidant capacity is in this report referred to as "bioactivity".

4 Results

4.1 pH swing method

Following the developed pH swing method (Figure 3), 2000 g mussels were processed. The separated supernatants from step 1 and step 2 were used in the filtration trial. As can be seen in Table 1 the pellet material is mostly insoluble and does not even dissolve at pH 10. The material that does dissolve at pH 10 was collected as precipitated material at pH 4. The material that was dissolved at neutral pH (supernatant 1 & 2) was collected, blended and used for the separation of the soluble fraction. Overall the amount of material that dissolves is low. This may indicate that a higher pH may be required, however a higher pH is not desired as it will lead to more protein damage, saponification of fats and a likely reduction of the bioactivity.

Table 1 mass balance of the pH swing method.

	Weight (g)	% DM	DM (g)
Mussels start (blanched)	2000		
Shells	1395		
Meat	605		
After blending	545		
Centrifuge step 1			
Supernatant 1	150	3.1	4.7
Pellet 1	392	18.5	72.5
Wash, centrifuge step 2 Input: 350 g pellet, 150 g water			
Supernatant 2	173	2.4	4.2
Pellet 2	321	20.9	67.1
pH 10 extraction, centrifuge step 3 Input: 275 g pellet, 150 g water, 7 g NaOH (1 M)			
Supernatant 3	169	2.9	4.9
Pellet 3	251	21.2	53.2
pH 4 precipitation, centrifuge step 4 Input: 150 g SN3, 11 g HCl (1 M)			
Supernatant 4	140	1.1	1.5
Pellet 4	12	24.7	3.0

The samples that were produced in this trial were assessed on their bioactivity (Table 2). The analysis was normalised by setting the uric acid IC50 at 0.50 mg/mL. In this way the current results are comparable to those of previous analyses. Very interesting in these results was that the soluble fractions are clearly more active compared to the insoluble fractions. Pellet 1 was the only fraction that still showed some activity, however this was most likely a result of the supernatant fraction that was still present in the unwashed pellet. After washing was performed , the pellet did not show bioactivity, defined as having a IC50 higher than 10 mg/mL. The supernatant fraction of with the most activity was supernatant 2: the sample after washing the pellet. Solubilizing the pellet at higher pH resulted in some more active product that solubilizes, although the activity was lower as compared to the supernatant 2 after washing. These results show that the active fraction is the soluble fraction at pH 6, which is a fraction that is likely to be discarded in a process in which shellfish meat is produced in a meat processing facility. The pellet fractions would then be used since this fraction contains the most protein. Therefore in a biorefinery concept high protein fractions and highly bioactive fractions could be separated, yielding two interesting products. Protein rich material for nutritional purposes and bioactive material to be used as a functional ingredient in food or aquafeed.

The extraction of additional protein at high pH may not be very beneficial since the amount of protein that is additionally extracted at high pH is very small, while the properties of the insoluble fraction will most likely be negatively influenced by oxidation, modification and cross-linking.

Table 2 Bioactivity of the pH swing fractions.

Sample	IC50 (mg/mL)
Supernatant 1	7.19
Supernatant 2	1.18
Supernatant 3	4.57
Supernatant 4	5.93
Pellet 1	4.88
Pellet 2	>10
Pellet 3	>10
Pellet 4	>10

4.2 Separation of soluble fraction

For the separation of the soluble fraction, the blended mixture of supernatant 1 and 2 (SN1 and SN2, Figure 2) was used. The pH of this sample was 6. In order to increase the amount of soluble material, the pH was adjusted to 10. This sample was stirred for 30 minutes at room temperature, followed by centrifugation.
After centrifugation, the supernatant was used for the separation by molecular size. For this, 300 kDa membrane filters incorporated in Greiner tubes were used. Multiple centrifugation runs were attempted but no liquid passed through the filters. The separation based on molecular size was ended unsuccessfully. It is likely that the presence of fat in the sample makes the filtration of the material very difficult.

4.3 Direct pH swing method

The mussel material was fractionated in a separate experiment using the direct pH-swing method. This method comprises less steps and potentially yields more soluble product, as the material that is soluble at pH 6 is most likely also soluble at pH 10 and will end up in this fraction. This method will therefore result in a fraction of soluble material, containing the sarcoplasmic protein fraction and a part of the myofibrillar protein that dissolves at pH 10. The remaining insoluble protein will end up in the pellet fraction. As can be observed in Table 3 this method increase the amount of solubilised material in the supernatant slightly compared to the previously described pH swing method.

Table 3 Mass balance of the direct pH swing method.

	Weight (g)	% DM	DM (g)
Mussels start	2000		
Shells	1662		
Meat	338		
Water added	750		
NaOH added	15		
After blending	1054		
	Centrifuge step 1		
Supernatant 1	812	1.9	15.4
Pellet 1	240	18.5	44.4
	Centrifuge step 2		
Supernatant 2			
Pellet 2			

The samples that were produced in this trial were assessed on their bioactivity (Table 4). In this analyses it appears, similar to the bioactivity assessment of the previously described pH swing method, that the solubilized material has a high bioactivity. The pellet material contains most of the mass and also most of the protein but is low in bioactivity. The overall results confirm the previous findings that the bioactivity is in the soluble fraction and most of the mass and protein is in the insoluble fraction. This is promising for envisaged production of fractions that are products for different markets.

Table 4 Bioactivity of fractions from direct pH swing method.

Sample	IC50 (mg/mL)
Blended mussels	4.16
Pellet 1	>10
Supernatant 1	2.51
Pellet 2	>10
Supernatant 2	1.85

4.4 Large scale extraction of bioactive fraction

This production trial was performed to produce enough material for allow a feeding trial using sole (Table 5). During this experiment samples were collected for bioactivity analyses and feed safety assessments. The trial was started with 300 kg of mussels. Mussels were delivered in 15 kg packages. Due to loss of moisture and some sampling, 262 kg of mussels were blanched in batches of approximately 5 kg. The blanching process lead to additional loss of moisture from the mussels. This amount could not be quantified, since this occurs in the blanching bath, which needs to be drained somewhat between the blanching steps. During the separation of the shells from the meat by hand, some material was collected for additional analyses, a batch of 5 kg was overcooked and closed and broken shells were sorted out, leading to a total loss of 45 kg of mussels. The first steps in the processing yielded a final 82.7 kg of blanched mussel meat and 134 kg of shells and 45 kg of loss in terms of moisture and broken and closed shells.

Table 5 Mass balance of the large scale extraction trial.

	Amount (kg)	% DM	DM (kg)	Protein (%dm)
Raw mussels (in)	262			
Blanched meat	82.7			
Shells	134			
Loss	45.3			
16.2 kg meat set aside, 66.5 kg milled to slurry				
set to pH 6, stir one hour				
Blanched meat	66.5			
Ground slurry	65.1			64.9
Pellet	36.5	21.6	7.9	83.1
Supernatant	27.7	9.6	2.7	46.1
Diluted supernatant	6.1	1.2	0.1	

16.2 kg blanched mussels were set aside and packed separately in 48 bags of 300 g meat each. The other 66.5 kg mussel meat was ground into a slurry (Figure 6). The pH of the slurry was adjusted to pH 6 and stirred for 30 minutes at room temperature to dissolve as much sarcoplasmic protein as possible. The material was subsequently centrifuged to separated dissolved and undissolved material. The tanks were rinsed with water and this water was also centrifuged to produce the material referred to as the diluted supernatant.

Figure 6 Grinding of the mussel meat into slurry.

As can be observed in the mass and protein figures of Table 5, the starting material has a protein content of 65%. The pellet is enriched in protein, because salts and oil are removed. The supernatant has a lower protein content compared to the ground slurry. The supernatant could be enriched in protein content by precipitation of the protein. The salts and oil are then removed. Filtration might be an option, but experiments have shown that filtration of this material is very difficult.

The products have been freeze dried and handed over, together with the bags with 16.2 kg blanched mussel meat, to Wageningen Livestock Research for the *in vivo* trials, after securing samples for the bioactivity assay. Samples from both small scale and large scale processing have been handed over to WSFR for food safety analyses. The bioactivity of the material has been analysed using the assay, developed in this project. The bioactivity was normalised and the IC50 of uric acid was set to 0.50 mg/mL. As can be observed in Table 6 the material that was produced had a very high bioactivity. Both pellet and supernatant had a low IC50, indicating the desired bioactive properties. The good result of the pellet may indicate that still a substantial amount of soluble and active material is present in the insoluble pellet. Washing these soluble components out of the pellet may lower the bioactivity of the pellet material, however on large scale this will most likely not be economically feasible. These results are very promising for the application of the material as feed ingredient in the *in vivo* trial in this project.

Table 6 Bioactivity of the samples from the large scale process

Sample	IC50 (mg/mL)
Mussel meat slurry	1.56
Supernatant	1.62
Pellet	1.73

Overall the method of protein production at neutral pH and separation of the fluid fraction yields two interesting products:

- A high protein pellet
- A highly bioactive supernatant

These results are very promising. In a process in which protein from marine low trophic sources such as shellfish is produced for application as nutritional or functional ingredient in food and feed,, a side stream containing a high bioactivity can be recovered, which can be directly applied as a aquafeed ingredient. In a future in which our protein sources will be partly covered by marine products, this is a very interesting and promising development.

5 Conclusions

- Bioactivity from pH swing methods is mostly found in the supernatant fractions, while not in the washed protein rich pellets
- The extracted material at pH 6 has a higher bioactivity compared to pH 10 extracted material.
- The separation of material at high pH yields only a small additional fraction, while the protein fraction becomes less interesting for food applications
- Large scale processing yields a solid fraction high in protein and a liquid fraction that is lower in protein. Both fractions display high levels of bioactivity.

The results of this study are very promising. In a process in which protein from marine low trophic sources such as shellfish is produced for application as nutritional or functional ingredient in food and feed, a side stream containing a high bioactivity can be recovered, which can be directly applied as a aquafeed ingredient. In a future in which our protein sources will be partly covered by marine products, this is a very interesting and promising development.

The material that is soluble at pH 6 appears to contain the highest bioactivity. pH 6 is very close to the biological pH of the material and therefore pH adjustment may not be required in the process. This can be beneficial as pH adjustment will also affect the pellet fraction, which may be a food product in the shellfish processing industry in the future.

6 Outlook

In 2023 the results of the *in vitro* trials are expected. These trials will determine to what extent the use of the extracts from mussels as bioactive feed ingredient do in fact help to avoid anaemia in sole. A separate project will be started up to study the current subject further.

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Report 2380

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