

RESEARCH REGARDING INTEGRAL PROCESSING OF MUSSELS FROM BLACK SEA

MAGDALENA MITITELU, CORINA IONIȚĂ*, ELENA MOROȘAN

Department of Clinical Laboratory and Food Safety, Faculty of Pharmacy, University of Medicine and Pharmacy "Carol Davila", Bucharest

** corresponding author: anacorina_ionita@yahoo.com*

Abstract

This paper presents a series of researches on *Mytilus galloprovincialis* L. species: the procedure for obtaining a lyophilised extract (from the whole mussels) followed by the assessment of its biological activity (pepsin inhibitory action, antimicrobial activity) and a technology for obtaining calcium lactate from the mussel shells followed by the analysis of the obtained calcium lactate, according to the methodology described in Romanian Pharmacopoeia Xth Edition (RPhX).

The success of the integral processing of mussels from the Black Sea for industrial application is sustained by the following factors: low price of raw material, accessibility and abundance of raw material, compliance with the requirements linked to the protection of the environment and acknowledged nutritious and therapeutic potential of the raw material.

Rezumat

În această lucrare sunt prezentate următoarele cercetări realizate pe specia *Mytilus galloprovincialis* L.: procedeul de obținere a unui extract liofilizat urmat de testarea activității biologice (acțiunea inhibitoare asupra pepsinei, acțiunea antimicrobiană) și o tehnologie de obținere a lactatului de calciu din cochilii de midii urmată de analiza lactatului de calciu obținut după metodologia din Farmacopeea Română Ediția a X-a.

Succesul tehnologiei de procesare integrală a midiilor din Marea Neagră la nivel industrial este susținut de următorii factori: prețul scăzut al materiei prime, accesibilitatea și abundența materiei prime, respectarea cerințelor legate de protecția mediului precum și de potențialul nutritiv terapeutic recunoscut al materiei prime.

Keywords: *Mytilus galloprovincialis* L., aqueous extract, pepsin inhibition activity, bacteriostatic action, calcium lactate.

Introduction

Mussels (*Mytilus galloprovincialis* L.) are an important source of proteins, biological active substances and calcium, therefore they can be used as food, because of the remarkable alimentary content, with high assimilation degree - 85% (30% proteins) [1, 2] and their shells can be used as calcium source for pharmaceutical use and as additive in animal food (92-94% of the calcium extracted from shells exists as carbonate salt) [2, 3, 4]. From the point of view of the quality of proteins, the mussels meat is not

inferior to the meat of domestic animals, furthermore, because of the presence of essential aminoacids (methionine, tyrosine, tryptophan), the proteins in the mussels overcome qualitatively the proteins in cattle and fish.

The paper presents the obtaining procedure of a rough extract from mussels and the determination of its main physical and biochemical characteristics and also a simple and efficient technology for extracting calcium lactate from mussel shells and the analysis of the obtained calcium salt.

The raw material (mussels from the Black Sea) represents one of the mass species in the Black Sea and on the Romanian seaside both by natural populations as well as cultured population. This natural resource from marine environment is not exploited in an organized manner and specialized at industrial level.

Materials and Methods

A lyophilised extract was obtained from samples of *Mytilus galloprovincialis* collected from the Romanian Black Sea Coast - Agigea and Cape Midia during august 2012 using two phases:

- 2.5 kg of washed mussels were treated with 2.5 L distilled water and boiled at 100°C for 60 min. in a recipient with ascending refrigerant (3.5 ± 0.1 L of aqueous extract and 1.5±0.2 kg of mussels were obtained after cooling and separating by decantation). The remaining mussels were separated from shells (0.3±0.5 kg of meat and 1.2±0.2 kg of shells were obtained); the meat was thrown and the shells were dried;
- the aqueous extract (3.5 ± 0.1 L resulted from the first extraction) and the separated meat (1.5±0.2 kg) were introduced in a recipient with ascending refrigerant and boiled at 100°C for 60 min.; after cooling, lipids were eliminated and the mixture was filtered under vacuum. The obtained filtrate (representing the rough extract of mussels) was concentrated by counter-dialysis with polyvinylpyrrolidone, then lyophilised.

The obtained lyophilised extract was divided into two parts: a first part (which was dissolved in distilled water, obtaining a 0.5% aqueous solution) and a second part used for establishing the main characteristics.

The bacteriostatic activity of a culture of *Escherichia coli* was analysed on a first part of lyophilised extract, using an alkaline gelose medium (pH=7.4–7.6) with the following composition (in g%): meat extract 0.3%; peptone 1%; NaCl 0.5%; agar 1.8%; distilled water 100 mL. On the medium surfaces, phenol solutions with different concentrations (1%; 0.5%; 0.3%; 0.1%) and 10% aqueous solution of lyophilised mussels extract were

added. After incubation at 37 °C, the bacteriostatic action of the lyophilised extract was determined by estimating the size of the inhibition zones, compared with phenol solutions. The determinations were made after 24 and 48 hours.

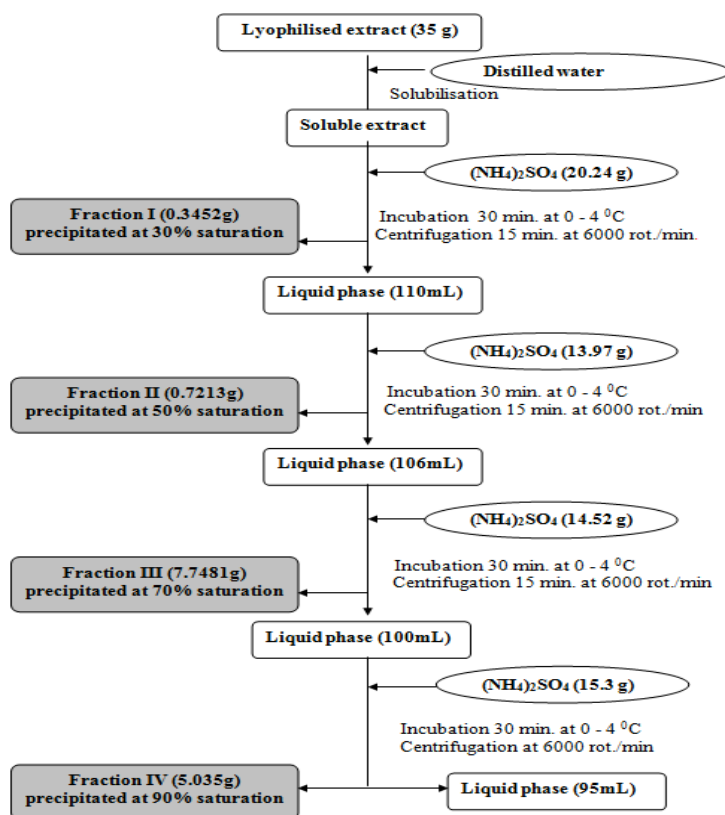


Figure 1

Fractionate precipitation of lyophilised mussel extract

The second part of the lyophilised extract was purified by saturation with $(\text{NH}_4)_2\text{SO}_4$ - it was used the procedure presented in figure 1, using the Dixon diagram [5]. Four fractions and a remaining liquid phase were obtained. Each of these fractions was concentrated by static dialysis (at 0 - 4 °C, for 48h). For each dialysate, there were tested:

- ✓ the enzymatic inhibitory action of a Pepsin standard solution Difco 0.1 mU Anson/mg (the determination of residual activity was realised according to RPhX [5, 6]),
- ✓ the loss on drying – according to RPhX [6],
- ✓ the total of glucides – using the method with orcinol [5].
- ✓ the total of lipids – using the technique of Christie [7],

- ✓ the total of proteins – using the technique of Lowry [8],
- ✓ electrical conductivity and pH measurements (using a *Radelkis* pH-meter-coductometer),
- ✓ spectral characteristics determinations (using a *Cecil 2501* UV-VIS spectrophotometer).

The technique for obtaining calcium lactate propose consists of the following steps [9, 10] (figure 2):

- ✓ chopping the raw material (1.2 ± 0.2 kg of dried shells remained from the first extraction grinded and pulverised in a mill with balls;
- ✓ removing the organic component, boiling the raw material with 1% KOH solution for one hour and filtration;
- ✓ washing: the mineral part was washed with distilled water (to eliminate the alkaline traces) and then dried;
- ✓ acid attack: the dried mineral part was submitted to lactic acid attack in a reaction bowl connected to vacuum pump, followed by filtration;
- ✓ purification: the resulted salt was purified by recrystallization from ethyl ether.

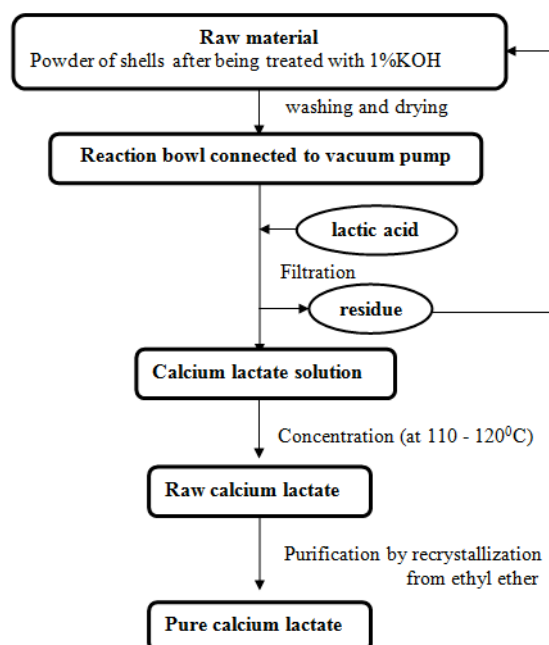


Figure 2

Technology for obtaining calcium lactate from the mussel shells

The obtained calcium lactate was analysed according to the RPhX: identification, quantitative determination, control of impurities, melting point [6].

The spectral characteristics of the obtained calcium lactate were determined using an UV-VIS *CECIL 2501* spectrometer and the obtained spectra were compared with the spectra of 1% solution of standard calcium lactate (prepared from Difco chemical pure substance).

Results and Discussion

In table I there are presented the results of pepsin inhibitory action on the four fractions obtained from fractionate precipitation of lyophilised mussel extract with different saturations in $(\text{NH}_4)_2\text{SO}_4$.

Table I
Pepsin inhibition activity of fractions analysed

Sample	Ratio inhibitor/enzyme(g/g)	Inhibition (%)
Fraction I	2.5	14.64
Fraction II	1.0	44.09
Fraction III	0.65	54.52
Fraction IV	0.62	69.53
Remained liquid phase	5.0	11.44

As it can be observed from table I, all fractions have a certain degree of pepsin inhibitory action, but fraction IV (corresponding for 90% saturation with ammonia sulphate) has the best inhibitory action.

- The main physical and biochemical characteristics of fraction IV and of the lyophilised extract of mussels are presented in table II.

Table II
Physical and biochemical characteristics of the lyophilised extract of mussels and of fraction IV (precipitate obtained after 90% saturation in $(\text{NH}_4)_2\text{SO}_4$ of the lyophilised extract)

Characteristic	Experimental results	
	For lyophilised total extract from mussels	For fraction IV
Aspect	white-yellowish power, hygroscopic	yellow-brown power, hygroscopic
Solubility	easy soluble in water, partially soluble in benzene and chloroform, insoluble in acetone	easy soluble in water, partially soluble in benzene and chloroform, insoluble in acetone
Total proteins (%)	38	60.5
Total glucides (%)	36	22.8
Total lipids	absent	absent
Electrical conductivity	2100 μS (for solution 0.1%)	2430 μS (for solution 0.5%)
pH	8.2 (for solution 0.1%)	7.04 (for solution 0.5%)
λ_{max} (absorption)	258 - 260 nm	275 - 280 nm
Loss on drying (%)	12.5	7.5

Table III presents the results concerning the action of the lyophilised extract on *Escherichia coli* culture by measuring the inhibition zones.

Table III
Size of inhibition zones

Sample	Inhibition zone (mm)	
	24h	48h
phenol 1%	8.5	5
phenol 0.5%	5.5	3
phenol 0.3%	3	1
phenol 0.1%	1	absent
10% solution of mussel extract	3	absent

Data from table III show that the lyophilised mussel extract has an “*in vitro*” bacteriostatic activity, which is comparable with that of phenol solution in concentration of 0.3%.

There are some observations concerning the technology for getting calcium lactate by treating the mineral part of mussel shells with lactic acid, followed by the purification of the salt using recrystallization from ethyl ether:

- the raw material is transformed into powder to increase the surface of contact and the efficacy of the reactive in use;
- the treatment by boiling with alkaline solution of KOH 1% is meant to release the organic component but it has the disadvantage of producing secondary reactions (protein hydrolysis for example);
- the mineral mixture - the washing of calcium carbonate must be realised under control of pH until the alkaline traces are eliminated (whose presence in the mass would impurify the product - the calcium lactate - and would increase the acid consumption);
- the reaction bowl was connected to the vacuum pumps to quicken the evacuation of carbon dioxide and ensure a certain level of the foam;
- the technology is simple and efficient (the efficacy of the reaction comparing to the consumed lactic acid was 85-90%).

The characteristics of the experimental by obtained calcium lactate and RPhX limits are given in table IV. As it can be observed from this table, the characteristics of calcium lactate obtained from mussel shells are included in the limits accepted by the RPhX.

Table IV
Characteristics of the obtained calcium lactate compared with Romanian
Pharmacopoeia limits

Characteristic	Romanian Pharmacopoeia limits	Characteristics of obtained calcium lactate
Aspect	• white power; • inodorous; • bitter taste.	• white power; • inodorous; • bitter taste.
Solubility	• soluble in 0.2 mL water; • insoluble in alcohol, chloroform and ether	• soluble in 0.2 mL water • insoluble in alcohol, chloroform and ether
Identification • ammonium oxalate 40g/L • with H₂SO₄ 100g/L and KMnO₄ 50g/L (at boiling)	• white precipitate soluble in mineral acids; • characteristic smell	• white precipitate soluble in mineral acids; • characteristic smell
Acidity • phenolphthalein • with NaOH 0.1 M	• clear and colourless; • pink.	• clear and colourless; • pink.
Impurities • Barium (acetic acid 300g/L; CaSO₄ sat. sol) • Chloride (etalon solution) • Iron (etalon solution) • Butyric acid (H₂SO₄ 100g/L at boiling)	• clear solution; • max. 0.01%; • max. 0.03%; • without smell of butyric acid.	• clear solution; • $\approx 9.899 \cdot 10^{-3}\%$; • $\approx 1.647 \cdot 10^{-2}\%$; • without smell of butyric acid.
Melting point	204 ⁰ C	203 ⁰ C
Loss on drying (120 ⁰ C at constant weight)	max. 30%	$\approx 27.7847\%$
Sulphate (etalon solution)	max. 0.01%	$\approx 6.293 \cdot 10^{-3}\%$
Assay (with Na ₂ EDTA)	99-102 %	99.5%

The spectral characteristics of the calcium lactate obtained from mussel shells in comparison with those of a standard calcium lactate solution 1% are presented in table V; as it can be observed; there is a strong similarity between the spectral characteristics of Difco calcium lactate and the experimentally obtained calcium lactate.

Table V
UV-Vis spectral characteristics of Difco and experimentally obtained calcium
lactate

$\lambda_{\text{max.}}$ (nm)	Absorption		Transmission	
	Difco calcium lactate	Experimentally obtained calcium lactate	Difco calcium lactate	Experimentally obtained calcium lactate
264-265	0.1974	0.2140	-	-
333-336	0.0645-0.0713	0.0546-0.0760	85.1	88.32
401-407	0.0288-0.0244	0.0299-0.0231	94.17	93.30
840-844	0.0020	0.0026	99.84	98.78

Conclusions

The mussels from the Romanian coast of the Black Sea, acknowledged as abundant marine organisms with a valuable therapeutic and nutritious potential could be used in pharmaceutical industry in order to obtain new medicinal products with a good efficiency and minimum side effects.

The mussel lyophilised extract has the following main characteristics: pH = 7.8; $\lambda_{\max}$ = 258-260 nm; protein content = 32%; glucidic content = 32%; bacteriostatic activity comparable with 0.3% phenol solution.

The fraction corresponding for 90% saturation with $(\text{NH}_4)_2\text{SO}_4$ has the following main characteristics: pH = 7.04; $\lambda_{\max}$ = 275-280 nm; protein content = 60.65%; glucidic content = 22.8%; pepsin inhibitory action = 69.53%.

The technology applied for obtaining calcium lactate from mussel shells is simple, efficient and cost effective. The obtained calcium lactate could be used in pharmaceutical practice and as additive to animal food because its analytical characteristics are included in the limits accepted by the Romanian Pharmacopoeia Xth Edition.

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