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Molecular Weight Determination of a Protease Extracted from *Mucor pusillus*: Comparison Methods

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Abstract

Mucor pepsin, a protease used in milk coagulation, is purified by ion-exchange and by molecular exclusion on Sephadex G100. The molecular weight (MW) is determined by polyacrylamide gel electrophoresis under denaturing conditions in presence of sodium dodecyl sulfate (SDS) and by molecular exclusion chromatography. Approximate evaluation of molecular mass was conducted by elution of known MW proteins (BSA: 67 kDa, pepsin: 35 kDa and trypsin: 23.8 kDa) on Sephadex G-100 under the same conditions as the experimental sample. The electrophoretic profile shows that the active fraction studied appears as a single homogeneous band (monomeric form). According to the curve calibration, the molecular mass of the coagulant fraction is about 48 kDa. For *Mucor*, the observed MW value seems to be enigmatic. However, this result is confirmed by a proteomic analysis with close MW values obtained using conventional techniques. The protease studied by the Scafold ver. Software 2.0 and the analysis of the protein similarities indicate a MW of 46 kDa and the protease sequence of 427 amino acids.

Keywords

Protease, Molecular Weight, Molecular Exclusion, Electrophoresis, Proteomics

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1. Introduction

Milk-clotting enzymes have an important role in the process of cheese maturation [1]. Microbial rennet appears to be more promising because its production is cheaper, biochemical diversity is greater, and genetic modification is easier [2]. Consequently, researchers focus on microbial MCEs (*milk-clotting enzymes*). The enzymes from some fungi such as *Rhizomucor*, *Mucor pusillus*, *Endothiaparasitica* and others microbial sources, have been reported [3] and some biochemical properties have been studied. Indeed, in the field of the analysis and characterization of bioactive molecules, molecular mass determination is essential in the identification of the molecule. In macromolecular organic chemistry (organic polymers: proteins, nucleic acids, viruses, etc.), the problem of the determination of the molecular mass is more complex. Indeed, these macromolecular substances have, in most cases, the size and relatively high molecular masses. In addition, with rare exceptions, macromolecular compounds exhibit a significant heterogeneity of mass defined by the role of the substance. For example, most *Bacillus* milk-clotting enzyme described in the literature give molecular size of 34 kDa [1] or 65 kDa [4].

The purpose of this article is to describe an approach to a fairly accurate assessment of the molecular mass of a protease from a culture of a strain of *Mucor pusillus* through the employment and subsequent three techniques.

2. Materials and Methods

2.1. Protocol for the Production of Protease

Protease that was to be the subject of the study of molecular mass was extracted from a fungal strain of *Mucor pusillus* referenced 953771, from in lyophilized form from the laboratory of Cryptogamy, the National Museum of Natural History in Paris. The transplanting of the strain was undertaken at the *Laboratory of Food Technology of the National Institute of Agronomy* (*Algeria*). The culture medium used was solid (surface fermentation), as advocated by Levadoux *et al*. [5]. It is composed of 60 g of wheat and 10 mL of 0.01% ammonium sulphate solution. After seeding, the boxes of Petri dishes containing malt agar medium, and their incubation at 37˚C for 5 days, the spores were released using a 0.1% tween 80 solution. The spore suspension was filtered on the sterile glass wool. The cellular exoenzyme was extracted with phosphate (0.02 M, pH 6) buffer.

2.2. Purification Technics

A double purification of the crude protease was made. This was achieved using an ion (anion exchanger) exchange chromatography followed by molecular exclusion on a Sephadex gel column. This was necessary for the study of protein homogeneity of protease and amino acids (proteomic analysis) sequence.

2.3. Comparative Methods of Assessment of the Molecular Weight of the Purified Protease

2.3.1. Molecular Exclusion Chromatography

Separation was achieved in decreasing order of molecular weight on the gel Sephadex G-100 with the help of a column (1 × 60 cm) Pharmacia calibrated by a phosphate buffer solution (0.02 M; pH 6). Aliquot 1.5 mL sample was eluted with the same buffer at a flow rate of 2 mL/h. Optical density reading was carried out at 280 nm. Fractions with a coagulant activity were collected, concentrated, and then kept. Approximate evaluation of molecular mass was conducted by elution of known molecular weight proteins (BSA: 67 kDa, pepsin: 35 kDa and trypsin: 23.8 kDa) on Sephadex G-100 column under the same conditions as our purified coagulase.

2.3.2. PAGE-SDS Electrophoresis

At each stage of purification, a sample was kept frozen or freeze-dried for electrophoresis according to Laemmli [6] in order to check homogeneity and the purity of the enzyme. The different samples and markers, volume to volume in the sample buffer, separated on a polyacrylamide gel which then formed a gel separation at 12% and a 5% concentration gel. The electrophoretic migration was achieved through a “Max Fill Bioblock Scientific” electrophoresis system under the following conditions: voltage: 250 V, 12% and an amperage gel separation: 74 mA, time: 1 h at 20˚C. Coagulase electrophoretic migration was performed parallel to that of known molecular weight markers (Phosphorylase: 94 kDa, bovine serum albumin: 67 kDa, ovalbumin: 43 kDa, carbonic anhydrase: 30 kDa, trypsin inhibitor: 20 kDa and lactalbumin: 14 kDa).

2.4. Proteomic Analysis

Sequencing of anticoagulant protease isolated from the *Mucor pusillus* strain study conducted at the centre of the CHU Laval Quebec Proteomics platform in Canada.

Digestion in gel proteins

The bands of interest extracted from gels were placed in 96-well plates then washed with distilled water. Enzymatic digestion with trypsin was performed using a Mass Prep liquid-handling robot (Waters, Milford, USA) according to the protocol described by Shevchenko *et al.* [7], with modifications suggested by Havlis *et al.* [8]. Proteins were reduced with 10 mM DTT and alkyl with 55 mM iodoacetamide. Trypsin digestion was performed using 105 mM modified porcine trypsin (Sequencing grade, Promega, Madison, WI) at 58˚C for 1 h. The products of digestion were extracted using a solution containing 1% formic acid and 2% acetonitrile followed by a 1% formic acid solution and 50% acetonitrile. The extracts were concentrated under vacuum and re-suspended in 8 µl of a solution containing 0.1% formic acid; 4 µl of extract are analysed by mass spectrometry.

2.4.1. Mass Spectrometry

Samples of peptides were separated by chromatography online liquid capillary nano scale phase reverse (RP-nanoLC) and analysed by electrospray mass spectrometry (ES-MS/MS). Peptides are separated through a Pico Frit column Bio Basic C18 column, 10 cm × 0.075 mm internal diameter (New Objective, Woburn, MA) with a linear gradient composed of 2% - 50% solvent B (acetonitrile 0.1% formic acid) in 30 min, 200 µl/min flow (obtained by splitting flow). The acquisition of mass spectra was carried out using the software Xcalibur (software version 2.0, on a scale of the spectrum between 400 and 2000 *m/z*) and analysed using the software Mascot (Matrix Science, London, UK; version 2.2.0).

2.4.2. Protein Identification

Protein identification was conducted in the Uniref 14.0 fungi (platform Proteomics CHU Laval) Bank Software Scaffold (version Scaffold 2-01, Proteome Software Inc., Portland, OR) which was used to validate the MS/MS spectra based on the identification of peptides and proteins. The identification of a single peptide protein should not be taken for granted. In general, it is necessary to identify at least two different peptides (with 95% probability and for each of the peptide scores) of the same protein to consider this protein as being present in the sample. The identified proteins by a single peptide or more than two peptides but with scores lower than 95% are present in the list for identification purposes only.

3. Results

The results obtained using the different techniques for evaluating the molecular weight of the studied protease confirm the use of chromatographic methods. The determination of the molecular weight by polyacrylamide gel electrophoresis in denaturing conditions, in the presence of SDS (**Figure 1**) and the approximate evaluation of

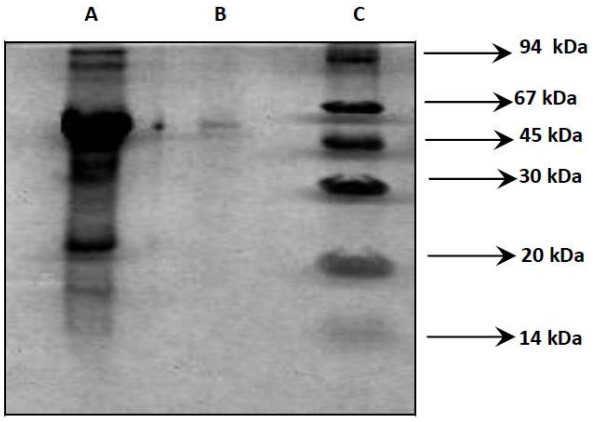

Figure 1. SDS-PAGE electro phoreotogram of *Mucor pusillus* extract. A: lyophilised crudeextract; B: purified extract on Sephadex G-100 filtration; C: standard molecular markers.

mass performed by the elution of known molecular weight proteins (BSA: 67 kDa, pepsin: 35 kDa and trypsin: 23.8 kDa) in Sephadex G-100 column (**Figure 2**) under the same conditions as the experimental sample (**Figure 3**) confirms our results. Molecular exclusion on Sephadex chromatography G-100 appears to be similar in the two methods.

The electrophoretic profile shows that the active fraction appears as one homogeneous band. Elution of purified protease produced a single peak of proteolytic activity. The value of PM obtained from the curve calibration markers to known weight and separated in the same conditions were in the order of 49 kDa. Although, the value of the obtained mass remains very high for a strain of the genus *Mucor* protease, it is clear that both techniques have analytical similarities by analysing proteomics with a PM.

In another component of the study, the protease sequencing (**Figure 4**) was conducted by a proteomic analysis in order to also determine the molecular mass of the protein. The study of protease using the Scaffold software and analysis of the similarities of proteins with a 95% probability of superior identifying did show a molecular weight of 46 kDa for a sequence of protease of 427 amino acids (**Figure 5** and **Table 1**).

4. Discussion

Proteomics, considered to be a very accurate technique in the analysis of proteins, confirms the results obtained

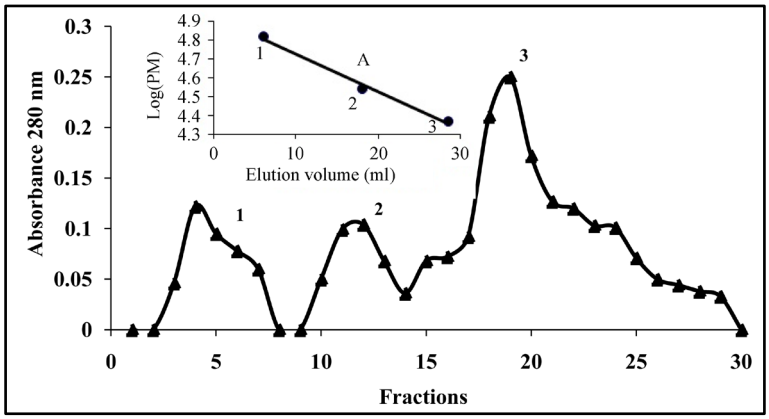

Figure 2. Gel filtration through Sephadex G-100 of standadr molecular markers (1) B.S.A.: 67 kDa; (2) pepsin: 35 kDa; (3) trypsin: 23.8 kDa. Pharmacia column (1 × 60 cm), elution bufer phosphate: (0.02 M; pH 6), flow rate: 2 mL/h, fraction: 1.5 mL. A: etalon curve of standard protein LogPM = f(Ve).

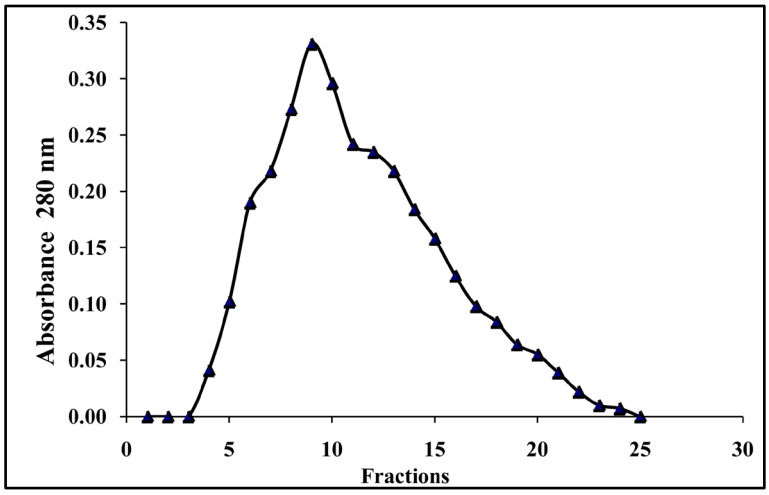

Figure 3. Gel filtration through Sephadex G-100 of *Mucor pusillus* (Pharmacia column 1 × 60 cm, elution buffer phosphate: 0.02 M; pH 6, flow rate: 2 mL/h, fraction: 1.5 mL).

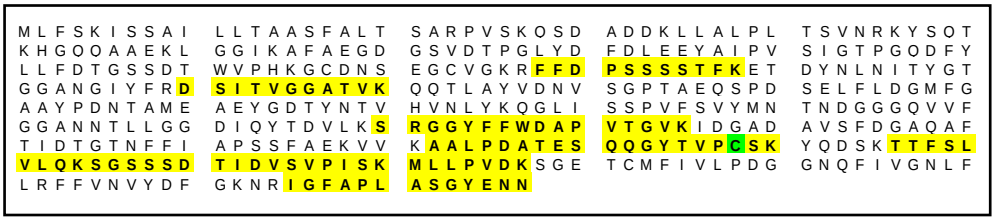

Figure 4. Amino acid sequence of the purified protease from *Mucor pusillus* (identification processing of proteins by Scafold Software 2.0).

index	peptide	exclusive to	valid	CARP RHIP P09177	CARP RHIP Q9UQ25
1	AALPDATESQQGYTVPCSK	CARP RHIP	☑	95%	95%
2	DSITVGGATVK	CARP RHIP	☑	95%	95%
3	FFDPSSSSTFK	CARP RHIP	☑	95%	95%
4	GGYFFWDAPVTGVK	CARP RHIP	☑	95%	95%
5	IGFAPLASGYENN	CARP RHIP	☑	95%	95%
6	MLLPVDK	CARP RHIP	☑	90%	90%
7	SGSSSDTIDVSVPISK	CARP RHIP	☑	95%	95%
8	SRGGYFFWDAPVTGVK	CARP RHIP	☑	87%	87%
9	TTFSLVLQK	CARP RHIP	☑	95%	95%

Figure 5. Identification of similar peptides (7 peptides) with probability at least 95% (yellow, disimilar peptides, for information only).

Table 1. Aminoacid sequence of the purified milk-clotting enzyme from *Mucor pusillus* and percentage of amino acids residue (%).

Aminoacids	Results		Amino acids	Results	
	Number of residue	Aminoacid residue (%)		Number of residue	Amino acid residue (%)
Alanine	33	7.72	Threonine	32	7.49
Valine	31	7.25	Cysteine	4	0.93
Leucine	31	7.25	Tyrosine	19	4.44
Isoleucine	18	4.21	Asparagine	20	4.68
Proline	19	4.44	Glutamine	17	3.98
Methionine	6	1.40	Aspartic acid	34	7.96
Phenylalanine	29	6.79	Glutamic acid	14	3.27
Tryptophan	2	0.46	Lysine	22	5.15
Glycine	45	10.53	Arginine	7	1.63
Serine	41	9.60	Histidine	3	0.70
Total				**427**	**100**

by conventional methods. With a difference in the order of 3 kDa or about 6 percent of the mass obtained with the SDS-Page and molecular exclusion, these results seem very close to that obtained with the other methods. Nevertheless, in commenting on these results, they do not coincide with those reported in the literature. According to Areces *et al.* [9] cited by Fernandez-Lahore *et al.* [10], fungal proteases are characterized usually by a molecular weight ranging from 32 to 34 kDa. This result is confirmed by some authors [1] [10]-[12], by studying the purified coagulases from *Mucor renninus*, *Penicilliumcaseicolum*, *Mucor* sp., and *Rhizopusoryzae*, respectively. Xiaoling *et al.* [3] reported that the purified enzyme from *Bacillus amyloliquefaciens* has a molecular mass of 58.2 kDa determined by SDS-Page, which is higher than those in the literature (34 - 49 kDa) for other microbial milk-clotting enzymes measured by gel filtration.

The molecular weight difference indicates that this feature depends on the origin of the coagulase, so further work on animal coagulases with reported molecular weight between 31 and 37 kDa [13] need to be performed. According to Garnot & Martin [14], chymosin and pepsin are characterized by a mass of 30 kDa and 35 kDa, respectively. Furthermore, the molecular weight in the order of 67 and 62 kDa has been reported for the coagulase of vegetable origin [15] [16].

5. Conclusions

The results obtained with the different techniques for evaluating the molecular weight of the protease confirm the use of chromatographic methods. The electrophoretic profile shows that the active fraction appears one homogeneous band and elution of purified protease gave a single peak of proteolytic activity. The value of PM obtained by the methods listed is in the order of 49 kDa. Although the value of the obtained mass remains very high for a strain of the genus *Mucor* protease, it is clear that both techniques have analytical similarities confirmed by a proteomic analysis with a molecular mass in the order of 46 kDa.

Proteomics, considered to be a very accurate technique in the analysis of proteins, confirms the results obtained by classical methods with the order of 3 kDa difference observed between the values of PM observed. Furthermore, this trial result could well be explained by a mutation of the strain in different experiments conducted on *Mucor pusillus*.

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