

# ZYMOGRAPHIC ANALYSIS OF METALLOPROTEASE AND CATHEPSIN IN STEATOTIC LIVER FROM MUSCOVY DUCK (*Cairina moschata*)

## Análisis zimográfico de metaloproteasas y catepsina en hígado graso de pato criollo (*Cairina moschata*)

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### ABSTRACT

Steatosis, the accumulation of triglycerides in hepatic cells after a rich in carbohydrate force-feeding, is a non pathological condition in palmipeds. The steatotic liver presents an enhanced metabolism. Protease activity in duck liver cells with steatosis was electrophoretically characterized. From exemplars of lean and fatty duck liver, three different homogenates were prepared: a plain homogenate, a metalloprotease extract and a cathepsin extract. The purpose of the study was to introduce a one-dimensional zymography (1DZ) and two-dimensional zymography (2DZ) approach in order to characterize the proteolytic profile present in hepatic cells under steatosis, with the ultimate goal to demonstrate the feasibility of this technique in determining proteolytic activity present in fatty liver in a semi-quantitative way. Protein concentration was 30% higher in fatty liver extracts. Employing casein as substrate, proteolytic activity measured at 280 nm was determined to be 25% higher in fatty liver extracts. Zymographic techniques allowed semi-quantitative results, successfully detecting cathepsin- and metalloprotease-activity using polyacrylamide gels copolymerized with gelatin and quantified by densitometry. The use of inhibitors confirmed the identity of the proteins studied. A metalloprotease activity was determined to have a relative molecular mass ( $M_r$ )  $\sim 90 \pm 5$  kDa, with an isoelectric point (pI) of  $5.55 \pm 0.01$ , and a cathepsin activity of  $38 \pm 2$  kDa with a pI of  $4.40 \pm 0.01$ . The results confirm an increase in these protease activities present in extracts from steatotic livers. The analysis of liver proteases activities in force-fed ducks may elucidate the mechanisms behind steatosis development.

**Key words:** *Cairina moschata*; cathepsin; electrophoresis; metalloprotease; steatosis; zymography.

### RESUMEN

La esteatosis hepática se define como una acumulación de triglicéridos en las células hepáticas, posterior a una dieta forzada rica en carbohidratos, y es una condición no patológica en palmípedos. El hígado esteatósico presenta una actividad metabólica mayor. La actividad proteásica en células de hígado de pato con esteatosis fue caracterizada por electroforesis. Tres tipos de homogeneizado (crudo, tratado para metaloproteasa y tratado para catepsina) fueron preparados a partir de ejemplares de hígado de pato, grasos y magros. El objetivo de este estudio fue introducir las técnicas de zimografía mono- y bi-dimensionales para caracterizar el perfil proteolítico presente en células hepáticas con esteatosis, con el fin de demostrar la factibilidad de esta técnica en determinar actividad proteolítica presente en hígado graso de manera semi-cuantitativa. La concentración proteica fue 30% mayor en extractos de hígado graso. Usando caseína como sustrato, la actividad proteolítica determinada a 280 nm fue 25% mayor en extractos de hígado graso. Las pruebas zimográficas empleadas permitieron resultados semi-cuantitativos, detectándose actividad catepsina y metaloproteasa usando geles de poliácridamida copolimerizados con gelatina y cuantificados por densitometría. El uso de inhibidores específicos confirmó la identidad de las proteínas bajo estudio. Se determinó una actividad metaloproteasa con  $M_r$   $\sim 90 \pm 5$  kDa y un pI de  $5,55 \pm 0,01$ ; y una actividad catepsina de  $38 \pm 2$  kDa con un pI de  $4,40 \pm 0,01$ . Los resultados confirmaron un aumento de estas actividades proteasas en los extractos de hígado esteatósico. El análisis de proteasas de hígado en patos con régimen de alimentación forzada pudiera elucidar el mecanismo involucrado en el desarrollo de la esteatosis.

**Palabras clave:** *Cairina moschata*; catepsina; electroforesis, metaloproteasas; esteatosis; zimografía

## INTRODUCTION

Fatty liver or steatosis is the accumulation of triglycerides in the hepatic cells, normally as the result of high-carbohydrate force-feeding [11]. Under biological stress, such a lipid excess exists as a response of the endoplasmatic reticulum, which includes the activation of proteins that causes resistance to insulin, caspase-4 mediated-apoptosis, inflammation and mitochondrial disfunction [1]. However, in palmipeds, the liver is where most fat is formed, being very sensible to fat degeneration, consisting in an abnormal fat reserve in the cytoplasm of parenchymatous cells. This is an anatomic consequence, given that the bird lacks a lymphatic system, fats from the feed goes to and store directly in the liver. Still, they possess an adipose tissue less able to synthesize fats, compared to mammals, thus being the liver the ultimate organ for fat metabolism [1,9]. The increase in liver weight is not due solely to the triglyceride accumulation (normally by a factor of 180), but also due to the increase of other components (proteins, phospholipids, cholesterol, DNA, RNA). Hence, in the particular case of palmipeds [i.e. species from the family of Anatidae, like ducks (e.g. *Cairina moschata*), or geese (e.g. *Anser* spp.)], a steatotic liver is not a pathological organ, but one that presents an enhanced metabolic activity; in contrast with the classical fatty liver pathological steatosis diagnosed in humans (fatty liver syndrome and fatty liver hemorrhagic syndrome).

Proteolytic activity has an important role in the regulation of a wide range of biological processes, given the high specificity associated to the hydrolysis of the peptide bond. The proteolytic mechanism regulates the action of a variety of proteins, and thus, also diverse cellular processes. This hydrolytic activity is given by a family of enzymes and zymogens present in the cell, called widely 'proteases', with the purpose to hydrolyze the peptide bond [7,21].

Several techniques have been used to determine the presence of proteases in biological samples. The use of electrophoresis for this purpose is a simple alternative widely use in the study of many types of proteases and its inhibitors [23,29]. Zymography is a valuable tool for the visualization of proteolytic activity on an electrophoretic gel matrix, by using a given protein substrate [25]. In order to characterize the proteolytic profile present in hepatic cells under steatosis, one-dimensional zymography (1DZ) and two-dimensional zymography (2DZ) approaches were introduced, with the goal to demonstrate the feasibility of this technique to determine proteolytic activity in fatty liver in a semi-quantitative way. For this, an extraction procedure was performed, commercial inhibitors were used to identify the type of protease, and the total proteolytic activity was quantified by photometry.

## MATERIAL AND METHODS

All experiments described fully comply with legislation on research involving animal subjects according to the European Communities Council Directive of November, 24 1986 (86/609/EC) [8], the Venezuelan Law of Science & Technology [16] and the Venezuelan Code of Bioethics and Biosecurity [17].

## Materials

2-mercaptoethanol, acetic acid, hydrochloric acid, phosphoric acid, casein, gelatin, tyrosine, 2-propanol, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA); Coomassie brilliant blue (CBB)-R250 and CBB-G250 were from Pierce (Rockford, IL, USA); sodium acetate, trichloroacetic acid (TCA), sodium chloride, calcium chloride, ethanol, sodium hydroxide, ammonium persulfate and tetramethylethylenediamine (TEMED) were purchased from Riedel de Häen (Seelze, Germany); glycerol, glycine, bisacrylamide, dithiothreitol (DTT) and Triton X-100 were from Promega (Madison, WI, USA). The electrophoresis mini-protean III model used was from Bio-Rad (Hercules, CA, USA). All chemicals used were of analytical grade, and deionized water was used.

## Biological samples

Three liver samples were collected from 3 Muscovy ducks (*Cairina moschata*) from different origins. The first sample was treated as reported by Awde *et al.* [4], and was designated as a positive control sample for fatty liver. Two other liver samples from Muscovy ducks were grown similarly until week 10. Then, one continued with regular feeding (negative control) and the other was force-fed until week 14. At this point, both birds were sacrificed. Livers were removed from carcasses, weighted, and samples were harvested, immediately frozen in liquid nitrogen and stored at -20°C (Whirlpool, Model ED2CHOXKO07, Whirlpool Corporation, USA) until analysis.

## Protein extraction

Three types of extracts were obtained from the three liver samples. First, an extract in buffer without inhibitors was designated as plain homogenate, second, an extract in buffer selective for metalloproteases [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 10 µg/mL (21 µM; MW 475.6) leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF)], and finally a cathepsin buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 20 mM ethylenediaminetetraacetic acid (EDTA)] [12]. For each extract, 400 mg were homogenized at 4 °C in a potter (Thomas Corning Potter-Elvehjem Tissue Grinder, 7725T-17, Thomas Scientific, USA) with the specified buffer. Afterwards, suspensions were centrifuged (Thermo Electron IEC Centra CL5R Refrigerated Centrifuge, ThermoFisher Scientific, USA) at 10,000 x *g* for 20 minutes (min) at 4 °C. Resulting supernatants were collected and stored at -20 °C (Whirlpool, Model ED2CHOXKO07, Whirlpool Corporation, USA) until assayed.

## Protein quantification

The total amount of protein was determined according to Bradford [6]. A calibration curve (triplicate) was plotted employing 1 g/L BSA as standard protein. Samples were assayed by duplicate; absorbance was measured at 595 nm (Genesys 10-S spectrophotometer, Thermo Scientific, Thermo Electron Corporation, USA).

### Proteolytic activity

A modification of the Kunitz [13] method was employed, with casein as substrate. Five hundred  $\mu\text{L}$  casein [1% (m/v) dissolved in 50 mM Tris-HCl pH 8.0] were preincubated at 37 °C for 15 min, then 200  $\mu\text{L}$  of the supernatant containing the enzymatic activity was added and the mixture was incubated at the same temperature with gentle and constant agitation for 20 min. Reaction was stopped by addition of 500  $\mu\text{L}$  TCA (5% m/v), an placed over ice for 30 min. Samples were centrifuged for 20 min at 2,500 x g. Finally, absorbance at 280 nm was measured to the resulting supernatant. Assays were done by triplicate. A negative control was performed by submitting TCA (5% m/v) directly to enzyme fraction and then the substrate was added. Enzymatic units were defined as the amount of  $\mu\text{mol}$  of tyrosine released as hydrolysis product from the casein per min. For this purpose, a calibration curve was plotted employing a 1 g/L tyrosine stock solution [19].

### Polyacrylamide gel electrophoresis

Electrophoresis was performed by a modified procedure described by Laemmli [14], under non-reducing conditions (i.e. absence of 2-mercaptoethanol, no sample heating). Unless elsewhere stated, 7.5 or 12% (m/v) resolving gels were used and 5% (m/v) as stacking gel. The Mini-Protean III (Bio-Rad) chamber was used. About 30  $\mu\text{g}$  protein dissolved in 4x sample loading buffer [Tris-HCl 200 mM pH 6.8, 4% (m/v) SDS, 40% (v/v) glycerol, 0.02% (m/v) bromophenol blue] were applied per well. Five  $\mu\text{L}$  of molecular weight standards (16.5-210 kDa range, ThermoScientific, USA) were used. Running buffer [Tris 25 mM, glycine 192 mM, 0.1% (m/v) SDS] was added to the chamber and electrophoresis was performed at 90 V the first 15 min, and then raised to 120 V, always at 4 °C, until the blue front reached the bottom of the gel. After the run, gels were stained with CBB-R250 [0.125% (m/v), 40% (v/v) 2-propanol, 10% (v/v) acetic acid] and destained [40% (v/v) 2-propanol, 10% (v/v) acetic acid] until optimal band patterns were observed. Digitalization of the gels was done with a transilluminator (Bio-Rad, Chemidoc, 1708280, USA) and images were saved as jpeg files. For molecular weight (MW) determination, a calibration curve with the standard proteins was performed, relating the log MW vs. relative mobility.

### Two-dimensional electrophoresis

For two-dimensional electrophoresis (2DE) purposes, a rehydration buffer [8 M urea, 1% (m/v) CHAPS, 40 mM DTT, 0.25% (v/v) Biolytes 3-10] was added to the liver extract (100-400  $\mu\text{g}$ ), avoiding to exceed 150  $\mu\text{L}$  of deposited sample. The immobilized pH gradient (IPG) strip was placed over the sample and left 30 min for absorption, then mineral oil was added. Strips were rehydrated under active conditions: 50 V, 19 °C, 11 h, without pause after rehydration, and with 50  $\mu\text{A}$ /strip. The voltage was programmed as follows: S1 200 V, linear, 1 h; S2 500 V, linear, 1 h, S3 1000 V, linear 1 h; S4 4000 V, linear 30 min, and S5 4000 V, ramping 5 h. After focusing, strips were

equilibrated for 20 min in equilibration buffer I [8 M urea, 0.375 M Tris pH 8.8, 2% (m/v) SDS, 20% (v/v) glycerol, 130 mM DTT] and then in equilibration buffer II [8 M urea, 0.375 M Tris pH 8.8, 2% (m/v) SDS, 20% (v/v) glycerol, 270 mM iodoacetamide (IAA)], and finally rinsed with water. The strips were then placed over the 10% (v/v) polyacrylamide gels, containing 0.15% (m/v) gelatin as substrate, and overlaid with heated agarose solution [1% (m/v) agarose, 0.025% (m/v) bromophenol blue] [28]. Gels were run as described before.

### Zymography

Metalloproteases and cathepsin activity was visualized by 1DZ and 2DZ. For metalloproteases, a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) copolymerized with 0.2% gelatin was pre-run in running buffer at 4 °C for 20 min at 125 V. About 50  $\mu\text{g}$  total proteins were applied and the system was run at 4 °C for 120 min at 125 V. After run, gels were incubated twice in renaturing buffer [100 mM glycine pH 8.0, 2.5% Triton X-100] for 30 min each. Then, solution was discarded and gel was placed in activating buffer [100 mM glycine pH 8.0] at 37 °C for 18 h. Finally, gels were stained with CBB-R250 0.5% (m/v) and destained until pale bands under a deep blue background were observed [10,27].

On the other hand, the zymography method proposed by Platt, Randall and Hanjoong [20] for cathepsin detection was used. PAGE (12.5% m/v) copolymerized with 0.2% (m/v) gelatin was run at 100 V, 4 °C, for 2 h. Sample buffer and running buffer were the same as previously described. Renaturing buffer [Tris 50 mM pH 7.4, 20% (v/v) glycerol] was used with mild agitation and then incubated with activation buffer [0.1 M sodium acetate pH 5.5, 1 mM EDTA, 2 mM DTT] at 37 °C for 18 hours (h). Afterwards, gels were briefly soaked in water (dd) and submitted to the same staining procedure previously described. For the 2DZ protocol, after the 2DE run, gels were treated with 1% (v/v) Triton-X 100, and then incubated in a suitable activation buffer [22,24]. Here, gels were washed and incubated with the same solutions used for the 1DZ.

### Proteolytic activity inhibition

To test the effect of specific inhibitors over the proteolytic activity, 200  $\mu\text{L}$  of enzyme samples were pre-incubated with inhibitor [10 mM EDTA, 20  $\mu\text{M}$  leupeptin or 1 mM PMSF] for 30 min at 37 °C, and then the photometric assay for activity determination was performed. The inhibition percentage was calculated against a control without inhibitor. Additionally, the effect of EDTA was visualized by zymography. Inhibitors were prepared as following: 30 mM EDTA (MW 372.24) stock solution. 0.0448 g EDTA disodium salt was dissolved in water, the pH adjusted to 7.4, final volume 4 mL. One mg/mL leupeptin (MW 475.59) stock solution was prepared in distilled water. 100 mM PMSF stock solution (MW 174.19) was prepared by weighing 0.0358 g and dissolving in 2-propanol in a final volume of 2 mL.

**Densitometry of zymograms**

The proteolytic activity detected by zymography was quantified according to band intensity, employing the Scion Image software, version BETA 4.0.2 (Scion Corporation) following the supplier's instructions. After desaturation of the image, colors were inverted, so that now black bands with a white background were obtained. Images were converted to .tiff files and imported to the software. Bands to be analyzed were defined and intensity was measured using the integral density modus. The data from the areas of the densitograms were plotted to compare the activity quantified in each band.

**RESULTS AND DISCUSSION**

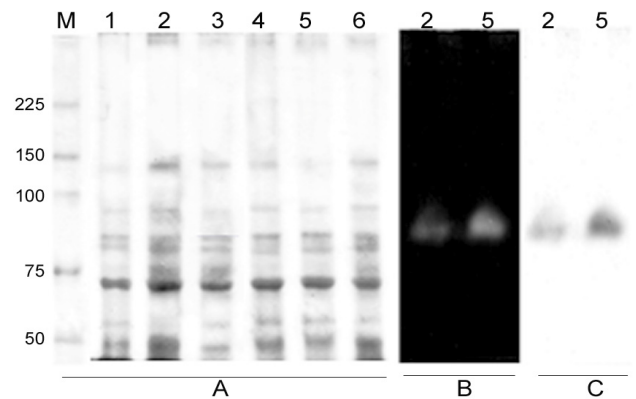
**Extraction and enzyme activity**

The different extraction methods employed were efficient for the separation of the different proteolytic activities present in the hepatic cells. Fatty liver samples were determined to be richer in total protein amounts, confirming previous reports indicating protein content increased by a factor of 1.6 in fatty liver compared with lean liver (TABLE I) [2]. All samples had protease activity, but those from fatty liver gave higher activity values. Metalloproteases and cathepsin extracts gave higher specific activities (TABLE I).

**SDS-PAGE and zymography**

The results of the analysis of the liver extracts on SDS-PAGE 7.5% (m/v) are shown in FIG. 1A. A ~90 kDa band was observed by zymography corresponding to metalloprotease activity (FIG. 1B). SDS is commonly used for electrophoresis, but is known to produce alterations in the protein tertiary structure, interrupting the interaction with the catalytic site of the protein. Thus, to maintain enzymatic activity, non-reducing conditions were employed. As result, the proteolytic profile of the sample was observed (FIG. 1B), and bands of the optimized color-inverted image (FIG. 1C) were analyzed by densitometry. Zymograms showed an active protease with molecular mass of ~90 kDa, present in the metalloprotease extract. The band intensity is ~100% higher in the sample from fatty liver, compared with the lean liver (calculations not shown). According to these results, it is proved that hepatic

cells under steatosis hold a higher metalloprotease activity. This result varies from the one previously reported [2], where a 72 kDa metalloprotease is detected. There may be a number of factors involved in this observation, as the method proposed here has several different features, including differences in activation buffer components, gel %, running conditions, and different incubation times. Although biological samples were treated similarly, geographic alterations may be present as well, as ducks raised in Venezuela may be metabolically distinct as those raised in France due to climate changes, diet variations, and duck farming techniques. For these reasons, comparisons between both works are limited. Beyond this, it has been reported strong variability in the processing yield of fatty liver from mule ducks, even if overfeeding and processing conditions are controlled [15].



**FIGURE 1. ELECTROPHEROGRAMS OF THE EXTRACTS AND ZYMOGRAPHY OF METALLOPROTEASES. A) SDS-PAGE (7.5%). LANES 1-3, EXTRACTS FROM LEAN LIVER; LANES 4-6 EXTRACTS FROM FATTY LIVER. LANES 1 AND 4, PLANE HOMOGENATE, 2 AND 5: METALLOPROTEASE, 3 AND 6: CATHEPSIN. B) ZYMOGRAM (7.5% AND 0.2% GELATIN) OF ONLY THE METALLOPROTEASE EXTRACT (100 MG). LANE 2, LEAN LIVER, 5: FATTY LIVER. C) COLOR-INVERTED IMAGE OF THE ZYMOGRAM SHOWN IN B). M, MOLECULAR WEIGHT MARKER (KDA).**

**TABLE I  
PROTEIN CONCENTRATION AND SPECIFIC ENZYMATIC ACTIVITY OF THE LIVER EXTRACTS**

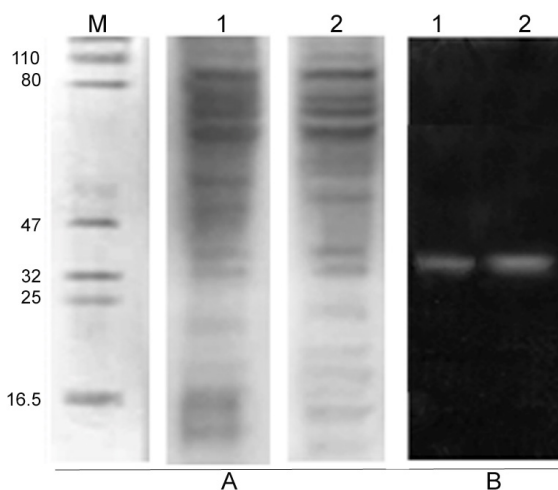
| Extract          | Protein concentration (mg/mL) |        |        | Specific activity <sup>a, b</sup> (U/mg) |           |            |
|------------------|-------------------------------|--------|--------|--|-----------|------------|
|                  | Liver sample                  |        |        | Liver sample                             |           |            |
|                  | Control                       | Lean   | Fatty  | Control                                  | Lean      | Fatty      |
| Plain homogenate | 31 ± 2                        | 42 ± 2 | 68 ± 2 | 1.7 ± 0.1                                | 1.4 ± 0.1 | 3.0 ± 0.1  |
| Metalloprotease  | 44 ± 1                        | 41 ± 1 | 61 ± 1 | 12.4 ± 0.3                               | 8.5 ± 0.3 | 20.5 ± 0.4 |
| Cathepsin        | 59 ± 2                        | 60 ± 2 | 79 ± 1 | 10.2 ± 0.1                               | 7.2 ± 0.3 | 21.5 ± 0.5 |

<sup>a</sup> Mean values from triplicates ± s.d.  
<sup>b</sup> U= μmoles tyrosine at 37 °C, 20 min



### Cathepsin zymography

Cathepsins belong to the cysteine protease family, and its presence was demonstrated in the samples analyzed. Former studies in animal models suggest that an increase in cathepsin activity have a potential implication in promoting adipogenesis and growth of adipose tissue [18]. FIG 2B shows the results obtained when lean and steatotic liver samples were loaded in 12.5% (m/v) zymograms. A ~37 kDa band containing cathepsin activity was detected, and was further corroborated by SDS-PAGE. Here is also detected an increase in proteolytic activity in samples from fatty liver, compared with lean liver. Differences in band intensity analyzed by densitometry gave ~100% increase in cathepsin activity in fatty livers (calculations not shown). This result is similar with that obtained by Awde *et al.* [2, 3], although they were able to see two extra bands of lower molecular mass, that were not detected with the method developed here. However, again, similarities are restricted for the same reasons explained formerly when discussing metalloproteases.

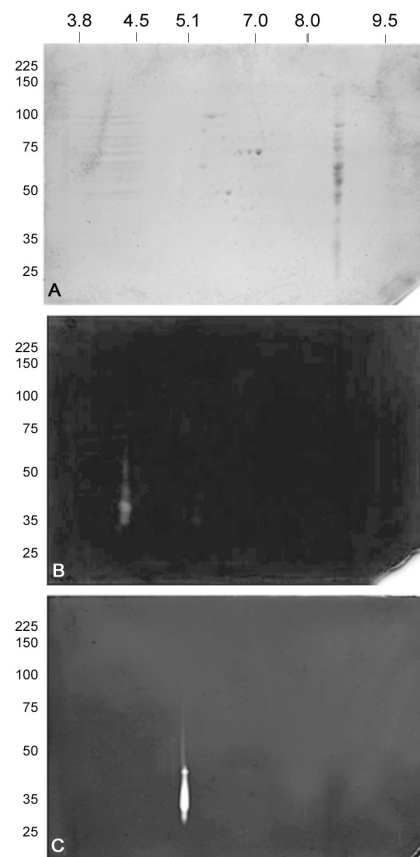


**FIGURE 2. ELECTROPHEROGRAMS FOR CATHEPSIN ANALYSIS. A) SDS-PAGE (12.5%), 30 MG OF TOTAL PROTEIN FROM CATHEPSIN EXTRACT WAS APPLIED. LANE 1: LEAN LIVER, 2: FATTY LIVER. B) ZYMOGRAPHY OF 80 MG CATHEPSIN EXTRACTS. LANE 1: LEAN LIVER; 2: FATTY LIVER. M, MOLECULAR WEIGHT MARKER (KDA).**

### Two-dimensional zymography

To further characterize the proteolytic activities, 2DZ was performed. As a first step, only the control liver was assayed. The electropherogram corresponding to 2DE analysis of the control liver is shown in FIG. 3A. Not many spots are present, but well defined protein spots are present in the region between  $pI$  5.1 and 7.0, and  $M_r$  50–100 kDa. A ladder pattern is found at  $pI$  ~8.5, and some weak bands are seen in the acidic region. For cathepsin activity (FIG. 3B) a middle intensity spot was obtained, indicating a  $pI$  ~4.4 and a  $M_r$  ~37 kDa, which agrees with the value obtained by 1DZ (FIG. 2B). Other weaker spots at the same  $pI$  are seen as well. On the other hand, metalloprotease activity analyzed by 2D

zymography (FIG. 3C) gave as result a  $pI$  ~5.5 and a smear with a  $M_r$  between 25 and 50 kDa. This new value strongly contrasts with the originally obtained by 1DZ, namely 90 kDa (FIG. 1B). Probably the harsh conditions employed during isoelectric focusing (IEF) might have driven to a protein denaturation, originating smaller polypeptide fragments. Similar values have been published [22], where several spots corresponding to cysteine protease had  $pI$  values in the range 4.8–6.3. Further assays were performed with the metalloproteins and cathepsin extracts from the lean and steatotic liver. However, reproducibility was an issue when comparing spots (data not shown). Though qualitative results may be given, difficulties were seen by enzyme proper activation (no spots on the gel), or deviations on the  $pI$  and/or  $MW$  were registered (harsh denaturing conditions proper of IEF, effect of urea, prolonged exposure to high voltage, etc.). It is advised to take care when submitting samples to IEF, as this type of variations had been reported before [30].



**FIGURE 3. ELECTROPHEROGRAMS FROM 2DE AND 2DZ OF THE CONTROL LIVER. THE FIRST DIMENSION WAS RUN WITH IEF STRIPS PH 3-10. SECOND DIMENSION WAS RUN WITH SDS-PAGE 10%. A) 2DE OF THE PROTEIN EXTRACT FROM LIVER CONTROL. B) 2DZ OF THE CATHEPSIN EXTRACT. C) 2DZ OF THE METALLOPROTEASE EXTRACT. IN EACH GEL ~250 MG TOTAL PROTEINS WERE LOADED.**

### Activity inhibition

EDTA, leupeptin and PMSF were tested to analyze its inhibitory effect over the proteolytic activities. The residual activity present in each sample was assayed by duplicates. Results are shown in TABLE II. It can be stated that no true inhibition was observed by any of the inhibitors when the cathepsin extract was used, about 37% of inhibition was calculated when treated with PMSF. True inhibition was detected when testing EDTA with the metalloprotease extracts of both liver samples (~90%). Bax *et al.*

[5] report an evolutive profile of proteins present in steatotic liver from duck. By coupling 2DE with Matrix-assisted laser desorption/ionization – time of flight - mass spectrometry (MALDI-TOF-MS), they identified 14 proteins, including proteases like calpains. This study may give rise to further analysis by submitting the resulting spots to mass spectrometry. A recent report describes the importance of employing MS technology to explain fat loss during cooking of *foie gras* and the fact that it is most likely associated with different proteolytic patterns [26].

TABLE II  
INHIBITOR EFFECT OVER THE PROTEOLYTIC ACTIVITY

| Extracts         | Inhibition <sup>a</sup> (%) |            |            |             |            |            |
|------------------|-----------------------------|------------|------------|-------------|------------|------------|
|                  | Lean liver                  |            |            | Fatty liver |            |            |
| Inhibitors       | EDTA                        | Leupeptin  | PMSF       | EDTA        | Leupeptin  | PMSF       |
| Plain homogenate | 28.2 ± 0.4                  | 13.4 ± 0.1 | 17.4 ± 0.1 | 21.4 ± 0.1  | 16.1 ± 0.4 | 15.0 ± 0.6 |
| Metalloprotease  | 90.1 ± 0.2                  | 6.9 ± 0.4  | 5.6 ± 0.5  | 89.4 ± 0.2  | 4.5 ± 0.3  | 3.0 ± 0.6  |
| Cathepsin        | 2.8 ± 0.3                   | 5.2 ± 0.1  | 37.2 ± 0.4 | 4.0 ± 0.2   | 6.4 ± 0.3  | 24.9 ± 0.3 |

<sup>a</sup> Mean values from duplicates ± s.d.

### CONCLUSIONS

The main producer of '*foie gras*' is France, being the fatty liver of ducks the most valuable product in these production systems. The quality determination of fatty liver is important for legal and economical reasons. Though variabilities in the analysis of fatty liver from ducks are expected to arise, even when overfeeding and processing conditions are controlled, comparisons among samples can be established. This investigation showed that all liver samples tested contained protease activity, albeit those from fatty liver gave a mean value of 2.5 times higher activity values when compared to lean liver samples. Metalloprotease activity showed a ~90 kDa band observed by 1DZ, but this value shifts when analyzed by 2DZ, obtaining a smear pattern with  $M_r$  25-50 kDa at  $pI$  ~5.5. On the other hand, cathepsin showed a ~37 kDa band during 1DZ, a value that differs from others published. Yet 2DZ analysis of cathepsin gave a spot corresponding to  $pI$  ~4.4 and a  $M_r$  ~37 kDa. Metalloprotease activity was inhibited in the presence of EDTA, however leupeptin failed to inhibit cathepsin activity. The protease analysis between the steatotic and non-steatotic liver was appreciated in this research, however further work must be performed in order to better characterize these activities, e.g. by MS. Recent publications further support the importance of this kind of research, where protease activities are screened to find paths responsible for quality losses during the cooking of ducks' fatty livers. Also semi-quantifications of proteins by electrophoresis and proteolytic activities by mono-dimensional zymography for metalloproteases and cathepsins have been

reported in order to study the effect of chilling rates on the quality features of fatty livers.

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