In vitro digestibility of protein with enzymatic crudes, obtained from residues of the slaughter house

Maryen Alberto[1], Lourdes Savón[1], Maday Alonso del Rivero[2] and Beatriz Farruggia[3]

[1]*Instituto de Ciencia Animal, San José de las Lajas, Apartado Postal 24, Mayabeque, Cuba*

[2]*Centro de Enzimas y Proteínas, Universidad de La Habana*

[3]*Laboratorio de Bioseparación, Departamento de Físico-Química, Universidad Nacional del Rosario, Argentina*

Correo electrónico: mvazquez@ica.co.cu

In order to study the *in vitro* digestibility of protein with enzymatic crudes enriched with pepsin substituting the commercial enzyme, residues from the experimental slaughter house of the Institute of Animal Science (ICA) were used. Two enzymatic crudes were obtained from 100 g of gastric mucus taken from bovine abomasum and porcine stomach of recently slaughtered animals. Both crudes were characterized using electrophoresis and zymography. The content of total proteins and enzymatic activity were determined. They were purified using polyethylene glycol (PEG)/phosphate two-phase systems. The proteolytic activity of crudes, and their purified fractions, was compared to the commercial pepsin through studies on *in vitro* digestibility of protein. A completely randomized design, with a 3 x 3 factorial arrangement was used, fixed in three treatments (commercial enzyme, porcine homogenate (HP) and bovine homogenate (HB)) and three times of incubation (2, 4 and 6 h). Crudes presented high concentration of total proteins (HP 27.19 $g.L^{-1}$ and HB 21.34$g.L^{-1}$) and high enzymatic activity, expressed in $g.L^{-1}$ of pepsin (HP 1.16 and HB 0.47). Pepsin within the crudes had a similar performance to the pattern enzyme of two-phase systems. It was concentrated in the superior phase, which was rich in PEG. Purification indicators for both crudes showed a purification factor superior to 3 and yields higher than 60 %. Digestibility analyses had no differences among treatments. It can be concluded that crudes and their purified fractions can be used for the analysis of *in vitro* digestibility of protein.

Key words: *crudes, pepsin, two-phase systems, in vitro digestibility*

In Cuba, there are few proteins that are isolated from a bacterial culture or purified from their natural source to supply the industry. Therefore, many of them are imported. The obtaining of enzymatic crudes for replacing commercial enzymes, for different uses, represents a considerable economical saving for the country. Besides, when the source for obtaining these enzymes is a residue, the benefit is higher because it includes beneficial secondary effects for the environment (Saval 2012).

According to Domínguez (2012), Cuba generates around 16 000 t of meat by-products from porcine and bovine slaughters. Currently, there are no efficient technologies for their processing. For a proper management of these residues, it is important to implement national and institutional policies, which will help to mitigate the growing problem of the emission of these wastes to the environment. The use of these wastes, not only as food or fertilizer, but also as a source for obtaining many enzymes, is a practical solution.

According to Arias and Lastra (2012), the enzymes from porcine and bovine pancreas (α-amylase, phospholipases, trypsin and chymotrypsin) are among the most commercialized enzymes. Pepsin is also widely commercialized, which is obtained from porcine and bovine gastric mucus. This one has a wide range of application, which includes its use in medical, pharmaceutical and food industries.

Among its main applications, pepsin is necessary for nutrition studies because it is used for *in vitro* reproductions of digestibility of many foods. Obtaining and purifying pepsin from wastes, for its further use in different processes, conveys an environmental benefit and an option for decreasing the deficit of imported enzymes.

The objective of this study was to obtain an enzymatic crude, enriched with pepsin from residues of the slaughter house, for its further use in studies of *in vitro* digestibility of protein.

Materials and Methods

Obtaining enzymatic crudes. The enzymatic crudes were obtained using slaughter wastes from the experimental slaughter house of the Institute of Animal Science, located at Mayabeque province, Cuba. A sample from the bovine abomasum mucus and another from porcine stomach, specifically from fundic area, were taken. Three animals were used in each case and a total of 100 g of mucus were collected. Samples were mixed in a blade homogenizer, with 300 mL of phosphate buffer (50 mM), according to the methodology proposed by Boeris *et al.* (2009).

The animals used consumed the same basal diet and belonged to the superior category.

Characterization of crudes. In order to determine the enzyme presence, a polyacrylamide gel electrophoresis at 12.5 % with SDS, and the comparison pattern used was a solution of commercial pepsin (1mg/mL). To determine the possible proteolytic activity of extracts, a zymography with gel (16 %) as substrate was used, included in a polyacrylamide gel at 15 %. Both studies were performed according to the methodology proposed

by Lantz and Cibroski (1994).

The protein concentration was determined in a spectrophotometer UV/VIS Optizen POP, through readings of absorbancy at 280 nm. An arbitrary molar extinction coefficient, equal to the unit, was used.

In order to quantify the amount of pepsin from crude extracts and from purified fractions, the technique of enzymatic activity by milk coagulation was used according to the methodology of Tubio *et al.* (2007). The enzymatic unit taken was the amount of enzymes that coagulates 2 mL of skimmed milk at 35 °C per minute

Activation of pepsinogen. It was carried out when the pH of the solution was decreased to a value of 2.5 through the slow adding of concentrated HCl. When this pH value was reached, it was kept at a constant temperature of 20 ºC and let to rest for 30 min. Later, the pH was increased to 6.4 by adding concentrated NaOH. It was centrifuged at 1500 rpm for five minutes, and the pellet was disregarded.

Extract purification. It was carried out through the separation of aqueous two-phase systems, formed by polyethylene glycol (PEG) with a molecular mass of 1500 and potassium phosphate (50 mM). Systems were prepared according to binodial diagrams, established by Lei *et al.* (1990) and Tubío *et al.* (2006). The distribution of pure pepsin in the two-phase systems was evaluated and later, the distribution of pepsin within HP and HB was determined.

The coefficient of pepsin distribution (Kpe) was determined as the relation of enzymatic activity in the superior and inferior phases, respectively. The coefficient of total protein distribution (KpPT) was determined as the relation of protein concentration in the superior and inferior phases, respectively.

Purification parameters of the process were determined. Yield (y%) in superior phase was calculated using the following equation:

$$y\,\% = \frac{100}{1 + \frac{1}{K_p R}}$$

Where: R- is the quotient of volumes from superior (Vsup) and inferior (Vinf) phases, respectively.

Purification factor was calculated according to the following equation:

$$FP = \frac{A_{sup} / [PT]_{sup}}{A_H / [PT]_H}$$

Where:

A_{sup} is the enzymatic activity from the superior phase of each system.

AH is the activity in the initial homogenate.

$[PT]_{sup}$ is the protein concentration from the superior phase of each system.

$[PT]_H$ is the protein concentration in the initial homogenate.

Determination of in vitro digestibility of protein. In order to evaluate the proteolytic capacity of crudes and their purified fractions, the technique of HCl pepsin digestibility, proposed by Furuya (1980), was used. The commercial soy meal (*Glycine max*) was used as protein food. Two experiments were performed for that purpose.

The first experiment consisted on the determination of *in vitro* digestibility of protein with the use of crude extracts. Three treatments were constituted (control with commercial pepsin, bovine crude extract and porcine crude extract) and three times of incubation were established (2, 4 and 6 h).

The second experiment consisted on the determination of *in vitro* digestibility of protein with the use of purified fractions of the extracts. Three treatments were constituted (control with commercial pepsin, purified fraction of bovine extract and purified fraction of porcine extract) and three times of incubation were also established (2, 4 and 6 h).

Experimental design and statistical analysis. For both experiments, a completely randomized design, with a 3 x 3 factorial arrangement was applied, formed by three treatments and three times of incubation. For the analysis of results, the computer statistical package InfoStat (Balzarini *et al.* 2001) version 5.1 on Windows XP was used. Mean values were compared using the test of Duncan (1955) in the necessary cases.

Results and Discussion

The results of the polyacrylamide gel electrophoresis with SDS showed, for both homogenates, clear bands in the region of 34 KDa, similar to the pepsin pattern (figures 1 and 2). This indicates the possible presence of pepsins in the samples. After the activation of extracts, these bands were intensified, associated to the existence of the enzyme, with the following disappearance of the band, related to the presence of the pepsinogen, around 42 KDa.

According to figure 3, both homogenates show proteolytic activity, even higher than the pattern of pepsin used in the study. The pepsinogen activation process increased the amount of active pepsin enzyme, and a higher proteolytic activity was observed, in the cases the extracts were activated. The porcine homogenate (HP) shows more marked bands than the bovine homogenate, which is associated to a higher concentration of pepsin in this extract.

Table 1 shows the protein concentration and enzymatic activity of crudes.

The proteolytic activity, measured as coagulase activity of milk, shows higher values in the case of HP, although both crudes show high proteolytic activity. Similar values of coagulase activity were reported by Boeris *et al.* (2009) and Romero (2012) for homogenates of bovine abomasum.

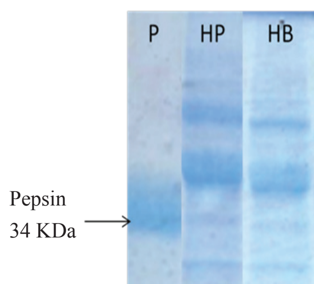

Figure 1. Polyacrylamide gel electrophoresis al 12.5 %
Line 1: pepsin pattern (1mg/mL)
Line 2: porcine homogenate (HP)
Line 3: bovine homogenate (HB)

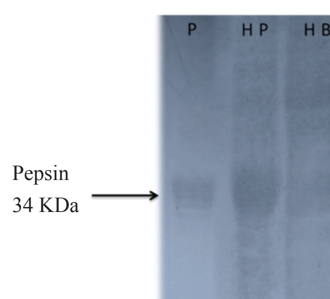

Figure 2. Polyacrylamide gel electrophoresis at12.5 %, after extract activation
Line 1: pepsin pattern (1mg/mL)
Line 2: activated porcine homogenate (HP)
Line 3: activated bovine homogenate (HB)

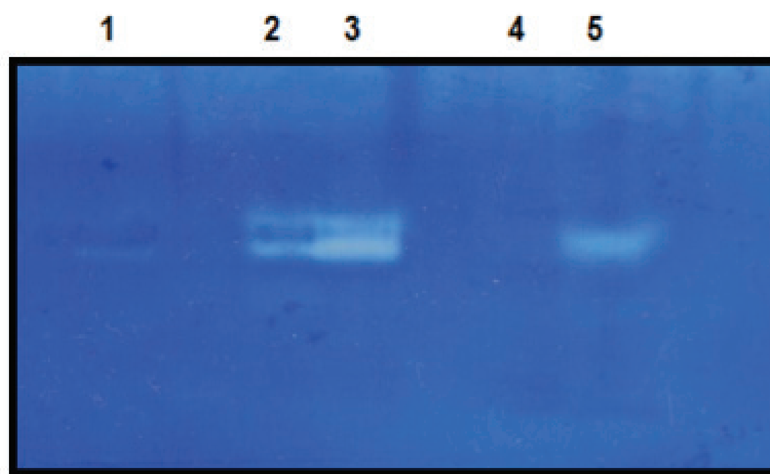

Figure 3. Zymography of activated and not activated homogenates, with gel (16 %) as substrate, included on a polyacrylamide gel at 15 %.
Line 1: pepsin pattern (1mg/mL)
Lines 2 and 3: not activated and activated HP, respectively
Lines 4 and 5: not activated and activated HB, respectively

Table 1. Protein concentration and enzymatic activity of porcine and bovine homogenates

Homogenates	Bovine	Porcine	SE ± Sig
Protein concentration ($g.L^{-1}$)	21.34	27.19	0.63***
Pepsin activity expressed in $g.L^{-1}$	0.47	1.16	0.03***

***P<0.001

The differences in the enzymatic activity between crudes of both species are mainly because of the differences in the process of food digestion in each case. According to studies performed by Álvarez *et al.* (2009), ruminant gastric juices, although having a similar composition to that of non-ruminants, have lower proteolytic activity and lower HCl secretion.

Purification of homogenates. The study of the distribution of pure pepsin in the two-phase system formed by PEG 1500/potassium phosphate showed a satisfactory separation of the enzyme towards the superior phase of the system, with a higher distribution coefficient than the unit. This indicates a favorable interaction between protein and PEG. According to studies of Imelio *et al.* (2008) and Spelzini *et al.* (2011), this performance of pepsin in the two-phase systems with PEG is mainly caused by superficial hydrophobicity of pepsin, because the great part of the hydrophobic residues of the molecule are located at the interior of the structure with high hydrophobic effect in the surface expoded to the solvent.

Figure 4 shows the purification parameters for each homogenate. The yield of enzymatic activity was higher than the protein yield in both homogenates, which indicates that the enzyme was mainly concentrated in the superior phase, full of PEG. Most of the impurities were located in the inferior phase and in the interface. Purification factors higher than 3 were achieved, which indicates that the control protein was extracted from a complex mixture.

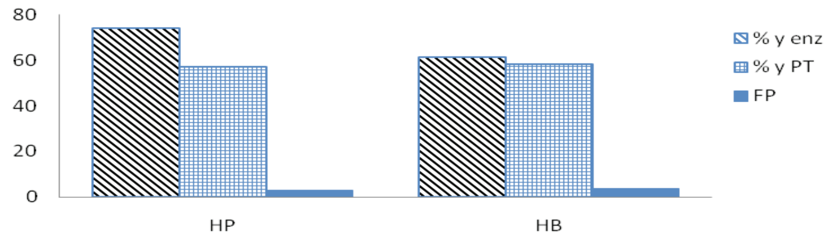

Figure 4. Yield of total proteins (% y PT), yield of enzymatic activity (%Yenz) and purification factor (FP) for porcine (HP) and bovine (HB) homogenates

Previous studies of pepsin purification, from its natural sources with the application of two-phase systems PEG/potassium phosphate, described by Boeris *et al.* (2009) and Palomares (2004), refer similar yield values and purification factors to this study.

It is known that the yield of a pure enzyme is very high (between 80 and 95 %). However, when the same method is used for the purification of the same enzyme within a complex mixture, like the homogenate of animal or plant tissue, the yield is low. This happens because of the presence of macromolecules, like proteins and nucleic acids, which interact among them and cause, many times, the precipitation of the enzyme in the interface (Bassani 2010). This study achieved yields between 60 and 75%, which indicates that great amount of enzyme was concentrated in the superior phase, only in one step of purification.

The use of aqueous two-phase systems for separating biomolecules, like enzymes, constitutes a useful method, mainly for obtaining enzymatic crudes that do not require high purity levels. This two-phase systems offer an adequate environment that preserves the biological activity of materials, and provides biomolecules with great stability. According to studies of Marini *et al.* (2011), aqueous two-phase systems provide a simple, fast and low-cost alternative and there is a possibility to apply them at large scale.

Determination of in vitro digestibility of protein. The study of digestibility with the use of non purified homogenates showed interaction between treatments and different times of incubation. Generally, there was a gradual increase of digestibility during the time of incubation. It is important to highlight that there were no differences between the commercial pepsin activity and the enzymatic activity of homogenates at 2 and 6h of incubation (table 2). This seems that there is a possibility of replacing the commercial product with the obtained natural crudes, because these last show higher activity in the foreseen period for digestion of nitrogen compounds, in non-ruminant species, according to Furuya (1980)

The study of digestibility with the use of the purified fractions of homogenates (table 3) also showed interaction between treatments and different times of incubation. There was a gradual increase of digestibility during the time of incubation. The highest value for all cases coincided at 6h. Nevertheless, there were no differences among treatments for the different times studied.

In both experiments, there was an increase of digestibility with the time of incubation in the non-purified homogenales as well as in the purified fractions.. According to Pedraza *et al.* (2012), this effect is a result of the food digestion process by the enzymatic action during the first hours, period in which the access of the enzyme to the substratum is more complex. However, once a part of it is digested, the proteolytic action is more favorable.

In the consulted literature, there are no studies of *in vitro* digestibility using enzymatic crudes, enriched with pepsin, obtained from wastes, or neither their purified

Table 2. *In vitro* digestibility using initial homogenates. Control treatment: commercial pepsin.

Variables	Treatment times	2	4	6	SE Sign
Protein digestibility	Control	74.86[ab]	71.22[a]	82.74[cd]	±1.27 P < 0.001
	HB	74.82[ab]	81.55[cd]	81.11[c]	
	HP	75.41[b]	79.69[c]	85.06[d]	

[abcd]Different letters indicate significant differences at P < 0.05 (Duncan 1955)

Table 3. *In vitro* digestibility using purified fractions of HP and HB

Variables	Treatment times	2	4	6	EE Sign
Protein digestibility	Control	76.30[a]	81.19[b]	83.02[c]	±1.12 P < 0.001
	HB	75.29[a]	77.40[ab]	82.88[c]	
	HP	76.27[a]	77.88[ab]	82.58[c]	

[abc]Different letters indicate significant differences at P < 0.05 (Duncan 1955)

fractions, instead of using pure enzyme. Nevertheless, this study suggests that crudes obtained from these wastes have a high pepsin concentration that can be used pure or without being purified. It is important to highlight that wastes from meat industry are an important source of enzymes. Therefore, they constitute an interesting resource for searching solutions to environmental contamination and deficient production of enzymes in Cuba.

Homogenates of gastric mucus from porcine stomach and bovine abomasum, as well as their purified fractions, can be used as substitutes of commercial pepsin in studies of *in vitro* digestibility of protein.

References

Álvarez, C. A., Pérez, H., De la Cruz Martín, T. M., Quincosa, J. & Sánchez, A. 2009. Fisiología Animal Aplicada. Ed. Félix Varela. Pp. 21-62

Arias, E. & Lastra, J. 2012. Tecnología enzimática. Available on:http://www.monografias.com/trabajos10/09tecenz/09tecenz.shtm. [Consulted: Octuber 8th, 2013]

Balzarini, M., Casanoves, F., Di Rienzo, J. A., González, L. A. & Robledo, C. W. 2001. Software estadístico: InfoStat, versión 5.1. Manual de usuario. Universidad Nacional de Córdoba, Argentina

Bassani, G., Fuciños, P., Picó, G. & Farruggia, B. 2010. *Candida rugosa* lipase Lip1–polyethyleneglycol interaction and the relation with its partition in aqueous two-phase systems. Colloids and Surfaces B: Biointerfaces. 75:532

Boeris, V., Spelzini, D., Picó, G. & Farruggia, B. 2009. Aqueous two-phase extraction and polyelectrolyte precipitation combination: A simple and economically technologies for pepsin isolation from bovine abomasum homogenate. Process Biochem. 44: 1260

Domínguez, J. C. 2012. Subproductos de la industria alimentaria que constituyen fuente de alimento animal. Informe del grupo empresarial de la industria alimentaria. Dirección de control productivo. La Habana, Cuba

Duncan, D. B. 1955. Multiple ranges and multiple F tests. Biometrics 11(1).

Furuya, S. 1980. A new *in vitro* method for estimating digestibility of animal feeds. JARQ. 14:52

Imelio, N., Marini, A., Spelzini, D., Picó, G. & Farruggia, B. 2008. Pepsin extraction from bovine stomach using aqueous two-phase systems: Molecular mechanism and influence of homogenate mass and phase volume ratio. Journal of Chromatography B. 873: 133

Lantz, M.S. & Ciborowski, P. 1994. Zymographic techniques for detection and characterization of microbial proteases. Methods Enzymol 235:563

Lei, X., Diamond, A. & Hsu, J. 1990. Equilibrium Phase Behavior of the Polyethilene glycol/Potassium Phosphate/water Two-Phase System at 4°C. J. Chem. Eng. Data. 35: 420

Marini, A., Imelio, N., Picó, G., Romanini, D. & Farruggia, B. 2011. Isolation of *Aspergillus niger* lipase from a solid culture medium with aqueous two-phase systems. J. Chromatography B. 879: 2135

Palomares, R. M. 2004. Practical application of aqueous two-phase partition to process development for the recovery of biological products. J. Chromatography B. 807: 3

Pedraza, R. M., La, O, Estévez, J, Guevara, G. y Martínez, S. 2012. Degradabilidad ruminal efectiva y digestibilidad intestinal *in vitro* del nitrógeno del follaje de leguminosas arbóreas tropicales. Pastos y Forrajes. 26:12

Romero, B. 2012. Desnaturalización térmica de la pepsina de cerdos. Master Thesis. Universidad Autónoma Metropolitana, México, D.F. Available on: ttp://148.206.53.231/UAM0663.PDF. [Consulted: November 12th, 2013]

Saval, S. 2012. Aprovechamiento de residuos agroindustriales: Pasado, presente y futuro. BioTecnología 16:15

Spelzini, D., Farruggia, B., Perez, N., Rua, M.L. & Pastrana, L. 2011. Production of aspartic peptidases by Aspergillus spp. using tuna cooked wastewater as nitrogen source and further extraction using aqueous two phase system. African J. Biotechnol. 10:15287

Tubio, G., Nerli, B. & Picó, G. 2007. Partitioning features of bovine trypsin and alpha-chymotrypsin in polyethyleneglycol sodium citrate aqueous two-phase systems. J. Chromatogr. B. 852-244

Received: May 21, 2014