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# Inhibitory activity of the angiotens in-converting enzyme from Chihuahua cheese-whey

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**Abstract**
The cheese industry in Mexico makes requeson cheese as an inexpensive way to concentrate whey protein. However, other alternative uses have been explored, such as ultrafiltration and diafiltration to produce liquid protein concentrates that are used in the preparation of beverages with or without lactose and with the angiotensin-converting enzyme (ACE) inhibitory activity. Prior to the use of whey, it is necessary to recognize its technological profile because the quality of the by-products that are produced depends on it. The objective was to evaluate protein content, proteolysis and the ACEinhibitory activity of the sweet whey obtained from the production of Chihuahua cheese, elaborated in four regions of Chihuahua state (Chihuahua, Delicias, Camargo and Cuauhtémoc). There were no significant differences in whey pH between regions. Cuauhtémoc and Camargo presented the highest protein concentration, 0.0469 and 0.0225 mM in the fractions <1 and <3 g/L, respectively. Camargo presented the highest degree of proteolysis (18.71 mM Gly), while there were no statistical differences in proteolysis between Chihuahua and Cuauhtémoc. The whey showed different levels of ACE inhibition, the highest IC50 (0.058 mg/mL) was recorded in the Delicias region.
**Keywords:**Angiotensin Converting Enzyme (ACE);whey; proteolysis;inhibitory activity;Chihuahua cheese.

**Resumen**
La industria quesera en México produce queso requesón como una forma económica de concentrar proteína de suero. Sin embargo, se han explorado otros usos alternativos, como la ultrafiltración y diafiltración para producir concentrados proteicos líquidos que se utilizan en la preparación de bebidas con o sin lactosa y con actividad inhibidora de la enzima convertidora de angiotensina (ECA). Previo al uso del lactosuero, es necesario conocer su perfil tecnológico porque de él depende la calidad de los subproductos que se produzcan. El objetivo fue evaluar el contenido de proteína, la actividad proteolítica y la actividad inhibidora de la ECA del suero dulce obtenido de la producción de queso Chihuahua, elaborado en cuatro regiones del estado de Chihuahua (Chihuahua, Delicias, Camargo y Cuauhtémoc). No hubo diferencias significativas en el pH del lactosuero entre las regiones. Cuauhtémoc y Camargo presentaron la mayor concentración de proteína, 0.0469 y 0.0225 mM en las fracciones <1 y <3 g/L, respectivamente. Camargo presentó el mayor grado de proteólisis (18.71 mM Gly), mientras que no hubo diferencias estadísticas en la proteólisis entre Chihuahua y Cuauhtémoc. El suero mostró diferentes niveles de inhibición de la ECA, la mayor IC50 (0.058 mg/mL) se registró en la región de Delicias.
**Palabras clave:** Enzima Convertidora de Angiotensina (ECA); lactosuero; proteólisis; actividad inhibitoria; queso Chihuahua.

## 1. Introduction

Hypertension is a risk factor for cardiovascular disease (CVD)(Kjeldsen, 2018). CVD is the leading cause of death worldwide, more people die yearly from CVD than from other illnesses, and from these more than three-quarters occur in low- and middle-income countries, even though most CVD are preventable(WHO, 2017). Hypertension is a risk factor for various illnesses such as coronary and valvular heart disease, left ventricular hypertrophy, cardiac arrhythmia including atrial fibrillation, stroke, and kidney disease(Kjeldsen, 2018).These pathologies can be treated pharmacologically and/or using natural products(Udenigwe& Mohan, 2014). Some dairy products have antihypertensive peptides that block the Angiotensin Converting Enzyme (ACE) (EC 3.4.15.1), which is an exopeptidase that cleaves dipeptides from the C-terminus. ACE is fundamental in the renin-angiotensin system, as it catalyses the conversion of angiotensin I into angiotensin II. Angiotensin II is a vasoconstrictor that increases

blood pressure(Ramírez-Rivas et al., 2022); hence, it needs to be inactivated, therefore any ACE inhibiting agent can be considered a coadjuvant against hypertension(Beldent et al., 1993; Donkor et al., 2007; Escudero et al., 2014).

Most of the whey produced by the cheese industry in Mexico is not used despite its high nutritional value and is, in many cases, thrown into the environment, thus generating a serious environmental problem due to the contamination of soils, aquifers and bodies of water(Mazorra-Manzano et al., 2019). Whey is a protein complex that positively impact health, therefore is used as a functional ingredient(Alvarado-Carrasco & Guerra, 2010). However, the functionality depends on the size and sequence of peptides that contain(Korhonen &Pihlanto, 2006). The above depend on the degree of proteolysis,which depends on the heat treatment applied to milk before curdling, type of rennet used during cheese making, inclusion or exclusion of starter cultures, ripening time, curdling pH, milk quality and whey storage conditions(Callejas-Hernández et al., 2012; Poveda, 2013). Proteolysis is caused by enzymatic activity and the rupture of small peptides that simultaneously release amino acids(Smit et al., 2005). It is essential in several aspects, it might determine the survival of the starter cultures, contributes to the formation of flavour and odour compounds, and bestow rheological properties to the fermented milk as well as allowing the formation of bioactive peptides(Serra et al., 2009). To date several studies have shown that dehydrated whey has an ACE-inhibitory action, but few studies have focused on the liquid form.The composition of the whey plays an important role because the quality of the by-products produced depends on it(Parra-Huertas, 2009).Therefore, the aim of this study was to determine protein content, proteolysis and the ACE inhibitory activity of sweet whey obtained from Chihuahua cheese process. The physicochemical and microbiological characterization of these whey´s have been reported previously (Paredes Montoya et al., 2014).

## 2. Materials and Methods

### *Whey Samples*

Sweet-whey samples (1 L) from Chihuahua cheese manufacture were collected from 25 dairies which belonged to four regions (Chihuahua, Delicias, Camargo and Cuauhtémoc), four samples (16 %) were discarded because these did not have ACE inhibition activity in none of the fractions (<3 kDa and <1 kDa). The regions were chosen because they highlight among the Chihuahua cheese production in Chihuahua state. Samples were transported to the laboratory of Biotechnology of Animal Foods in chilled containers and stored at -20 ° C until further analysis. The physicochemical and microbiological composition of the sera have been previously reported (Paredes-Montoya et al., 2014).

### *Protein content and hydrogen potential*

Protein content was determined by AOAC, (1984)991.20 method; and the hydrogen potential was determined by the AOAC (1947) 947.05 method, with a potentiometer (Hanna HI 98129, Hanna Instruments, Woonsocket, RI, USA). All analyses were performed in triplicate.

### *Proteolytic activity*

The proteolytic activity was performed according toDonkor et al., (2007). Briefly, 2.5 mL of whey and 5 mL of trichloroacetic acid (TCA) 0.75% were mixed and filtered (Whatman No. 1). Then, 50 µL of the filtrate and 1 mL of o-phthaldialdehido (OPA) and β-mercaptoethanol were mixed and incubated (room temperature) in a quartz cell. After two minutes the absorbance (340 nm) was measured in a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The degree of proteolysis was determined using a glycine (Gly) curve (y=0.0505x + 0.0638, R2=0.9977) and expressed as a mM of Gly.

### *Water-soluble protein fractions*

Whey aliquots of 250 mL were centrifuged at 12,000 g for 40 min at 5°C (Beckman Coulter, Fullerton, CA, USA). The supernatant was filtered through a filter (Whatman No. 40). The filtrate was fractionated (<3 kDa <1 kDa) by passing it through a membrane MilliporUltracel System® (Millipore Corporation, Billerica, MA, USA). Fractions were stored at -80°C until further. analysis.

### *Determination of ACE inhibitory activity*

The ACE inhibitory activity of the extracted peptide fractions was determined (<3 kDa <1 kDa) by RP-HPLC (high performance liquid chromatography resolution reverse phase) according to Pritchard et al., (2010)andGhassem et al., (2011), with some modifications. One unit of Commercial ACE (Sigma-Aldrich) was diluted in 50 mM Tris-HCl (pH = 7.5) with 0.3 M NaCl to obtain a concentration of 100 mU/mL. Then, 1 mL aliquots were stored at -20°C until use.Then, 50 µL aliquot of each fraction (<3 kDa or <1 kDa) or HPLC grade water in the case of the control, were mixed with 200 µL of 2.5 mM solution of Hippuryl-L-histidyl-L-Leucine (HHL) and the mixture was incubated at 37 ° C with shaking (450 rpm) for 30 min. To stop the reaction, 250 µL of 1M HCl were added. To separate the reaction product and the hippuric acid (HA) from the HHL, the mix was filtered through Whatman nylon filters (30 mm/0.20 µm). The filtrate was injected into a HPLC- UV-visible (Shimadzu, Kyoto, Japan) coupled with a

DiscoveryR C18 column with a 25 cm x 4.6 mm and 5 μm particle size (Supelco, Bellefonte, PA, USA) and eluted with 50-50% (v/v) water-methanol plus 0.1% (v/v) trifluoroacetic acid (TFA) at a flow rate of 0.4 mL/min and an absorbance of 228 nm. The evaluation of ACE inhibition was performed based on the comparison between the concentration of HA in the absence of the inhibitor (MC) and with it. For each peak, the area under the curve was determined, according to the HA obtained. The mean value of two determinations was measured to calculate the percentage inhibition of ACE using Eq. (1).

**Inhibition of ACE (%)** = (B – A/B – C) x 100 (1)

where A is the relative area of the peak generated by HA in the presence of the ACE inhibitor, B is the relative area of HA obtained in the absence of the inhibitor and C is the relative area of HA generated without ACE (corresponding to autolysis of HHL during the reaction). FBs contained a certain degree of HA, since this is naturally found in milk, so the HA concentrations of each sample were quantified to subtract them from the HA generated by the reaction and calculate the ACE inhibition percentage.

Additionally, peptide concentration and ACE-inhibitory activity (IC50) were estimated for each sample according toQuirós et al., (2005).

***Statistical analysis***

All data wereanalyzed by an ANOVA and process one fixed effect factor, with a significance level of $\alpha = 0.05$. Significant differences between regions were determined by a mean analysis using a Tukey test. Data were analyzed with SAS (2006 - 9.1.3; SAS Institute Inc., Cary, NC, USA).

## 3. Results and Discussion

### *3.1. Protein content and hydrogen potential of the whey*

Whey is classified according to its pH, as a sweet whey when pH is above 5.6 and as an acid whey when it is below 5.6(Callejas-Hernández et al., 2012; Poveda, 2013; Ramírez-Rivas & Chávez-Martínez, 2017). In this study, whey pH (table 1) ranged between 5.46 ±1.1 and 6.33 ±0.5, without statistical differences(P> 0.05) among regions.Calero et al., (2018) and Giroux et al., (2018)also reported pH values of 6.0 and 4.8 in sweet and acid whey, respectively. In the other handTarango-Hernández et al., (2015)obtained pH values of 5.0, 5.2 and 5.9 in fresh, ranchero and panela cheese.The conformation of proteins and their enzymatic activity is determined by the pH(Walstra, 2001), hence, pH fluctuation changes the structure and conformation of the proteins (α-lactalbumin and β-lactoglobulin)(Faryabi et al., 2008). There were no statistical differences(P> 0.05) in the content of protein(table 1) between regions. The average protein content of the whey was 7.2 g/L, which is within the range of 6 to 10 g/L reported byTunick, (2008). The protein content of the <1 kDa fraction varied between 0.016 to 0.047 g/L, a lower protein concentration was observed in the <3 g/L fraction (0.011 and 0.022 g/L).The <1 kDa fraction from Chihuahua had the lowest (P<0.05) protein content (0.0165 ±0.007 g/L); while there were no significant differences among Camargo, Delicias and Cuauhtémoc (P> 0.05). The fraction <3 kDa from Camargo had the highest (P<0.05) protein content (0.022 g/L), meanwhile the other regions did not have differences (P> 0.05). Variations in the protein content might be associated with the differences within the cheese production, or to the physicochemical composition of the milk(Tunick, 2008).

**Table 1.** *Physicochemical characteristics of whey derived the production of Chihuahua cheese.*
**Tabla 1.** *Características fisicoquímicas de sueros provenientes de la producción de queso Chihuahua.*

| | **Chihuahua (n=3)** | **Delicias (n=11)** | **Camargo (n=4)** | **Cuauhtémoc (n=3)** |
|---|---|---|---|---|
| Fosfatasa alcalina (% positive) | 0 | $6^{b}$ | $3^{a}$ | $3^{a}$ |
| pH | $5.460 \pm 1.100^{a}$ | $5.500 \pm 0.960^{a}$ | $6.040 \pm 0.620^{a}$ | $6.330 \pm 0.500^{a}$ |
| Proteolysis mM (Gly) | $15.380 \pm 300^{a}$ | $13.630 \pm 2.630^{b}$ | $18.710 \pm 4.330^{c}$ | $15.470 \pm 1.840^{a}$ |
| Protein <1 g/L | $0.016 \pm 0.007^{b}$ | $0.029 \pm 0.020^{a}$ | $0.046 \pm 0.001^{a}$ | $0.047 \pm 0.005^{a}$ |
| Protein <3 g/L | $0.013 \pm 0.009^{a}$ | $0.016 \pm 0.017^{a}$ | $0.022 \pm 0.002^{b}$ | $0.011 \pm 0.016^{a}$ |
| ACE inhibition <1a | $53.950 \pm 0.230^{a}$ | $57.470 \pm 38.840^{a}$ | $71.050 \pm 22.480^{b}$ | $51.070 \pm 12.690^{a}$ |
| ACE inhibition <3a | $27.050 \pm 13.850^{a}$ | $56.160 \pm 16.030^{a}$ | $82.750 \pm 48.150^{b}$ | $57.770 \pm 27.110^{a}$ |

[abc] Values with different superscripts between rows indicate significant difference P < 0.05.
ACE = angiotensin converting enzyme.
[a] Expressed in percentage.
[abc]Los valores con superíndices diferentes entre filas indican diferencia significativa P < 0.05.
ACE= Enzima convertidora angiotensina.
[a]Expresada en porcentaje

### *3.2. Proteolytic activity*

There were statistical differences($P < 0.05$) in the proteolysis (figure 1) of whey. Camargo had the highest degree of proteolysis (25.01 mM Gly); while Delicias showed the lowest (7.51 mM Gly). The variation of the proteolysis may be due to the starter culture used in the manufacture of Chihuahua cheese(Smit et al., 2005). The proteolytic system of the starter cultures is fundamental in the fermentation of milk, since it varies among microorganisms(Serra et al., 2009).

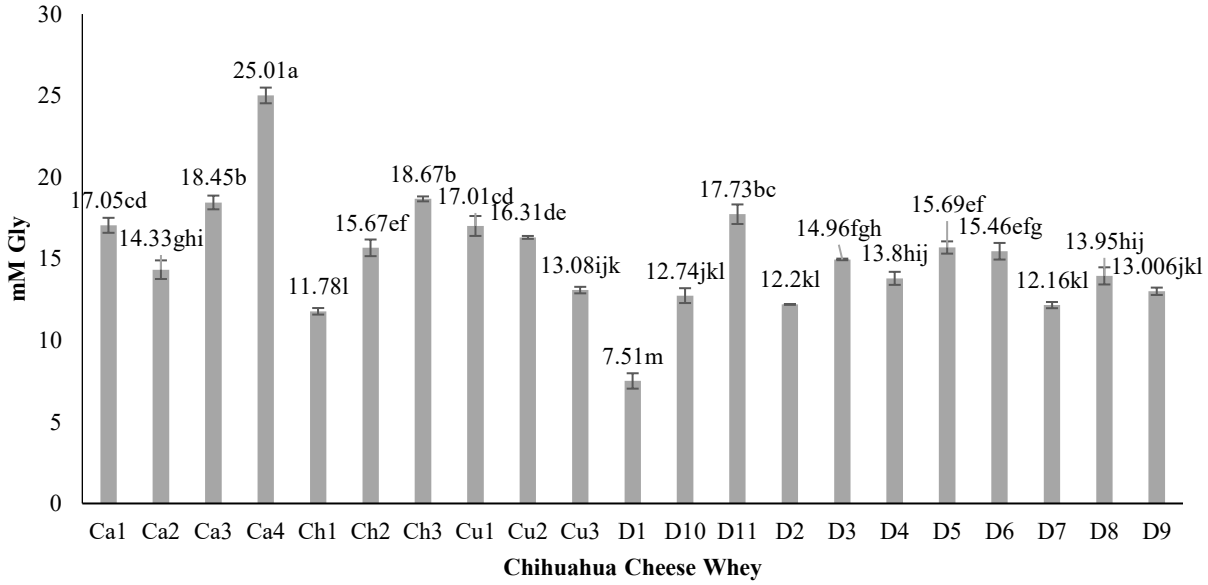


**Figure 1.**Proteolysis of Chihuahua cheese whey from different regions (Ca=Camargo; Ch= Chihuahua; Cu= Cuauhtémoc; and D=Delicias).

**Figura 1.**Proteólisis de suero de queso Chihuahua de diferentes regiones(Ca=Camargo; Ch= Chihuahua; Cu= Cuauhtémoc; y D=Delicias).

### *3.3. ACE inhibitory activity*

All regions and fractions indicated ACE inhibitory activity (figure 2, a and b), the values were expressed as half maximum inhibitory concentration IC50 (figure 3, a and b), however, the <1 kDa fraction had higher values. Tarango-Hernández et al., (2015)also showed the same thing, that at all fractions of whey from fresh cheese had ACE inhibitory activity.ACE inhibition ranged from 0.0133 ±0.002 to 0.1000 ±0.002 mg/mL and 0.0026 ±0.003 to 0.0500 ±0.005 mg/mL for fractions <1 and <3, respectively. Delicias (0.1 ±0.002 mg/mL) and Cuauhtémoc (0.05 ±0.005 mg/mL) had the highest ACE inhibitory activity in fractions <1 ($P<0.05$) and <3 ($P <0.005$), respectively. Conversely, Chihuahua had the lowest inhibitory activity; 0.013 ±0.003 mg/mL for the <1 fraction and 0.002 ±0.001 mg/mL for the <3. Likewise, it was observed that the whey with the highest ACE inhibitory activity had the greatest proteolytic activity (table 1, figure 2, and figure 3).Different ACE inhibition values have been reported(Tarango-Hernández et al., 2015)obtained a minimum inhibitory concentration between 0.57 and 4.52 μg/L, in whey from panela, fresh and ranchero cheeses.A wider range (between 5.2 and 10.7 mg/L) was observed in fresh cheese whey(Rodríguez-Figueroa et al., 2012)had similar ranges (0.034 and 0.61μg/L) with peptides released from milk after a Lactococcus lactis fermentation. The wide range of IC50, coincides with the variability reported in the present study. The differences might be linked to the size and sequence of the peptides during the manufacture of chesse(Korhonen &Pihlanto, 2006)mentioned that the percentage of ACE inhibition is determined by the hydrolysis and the amino acids and not the protein concentration.

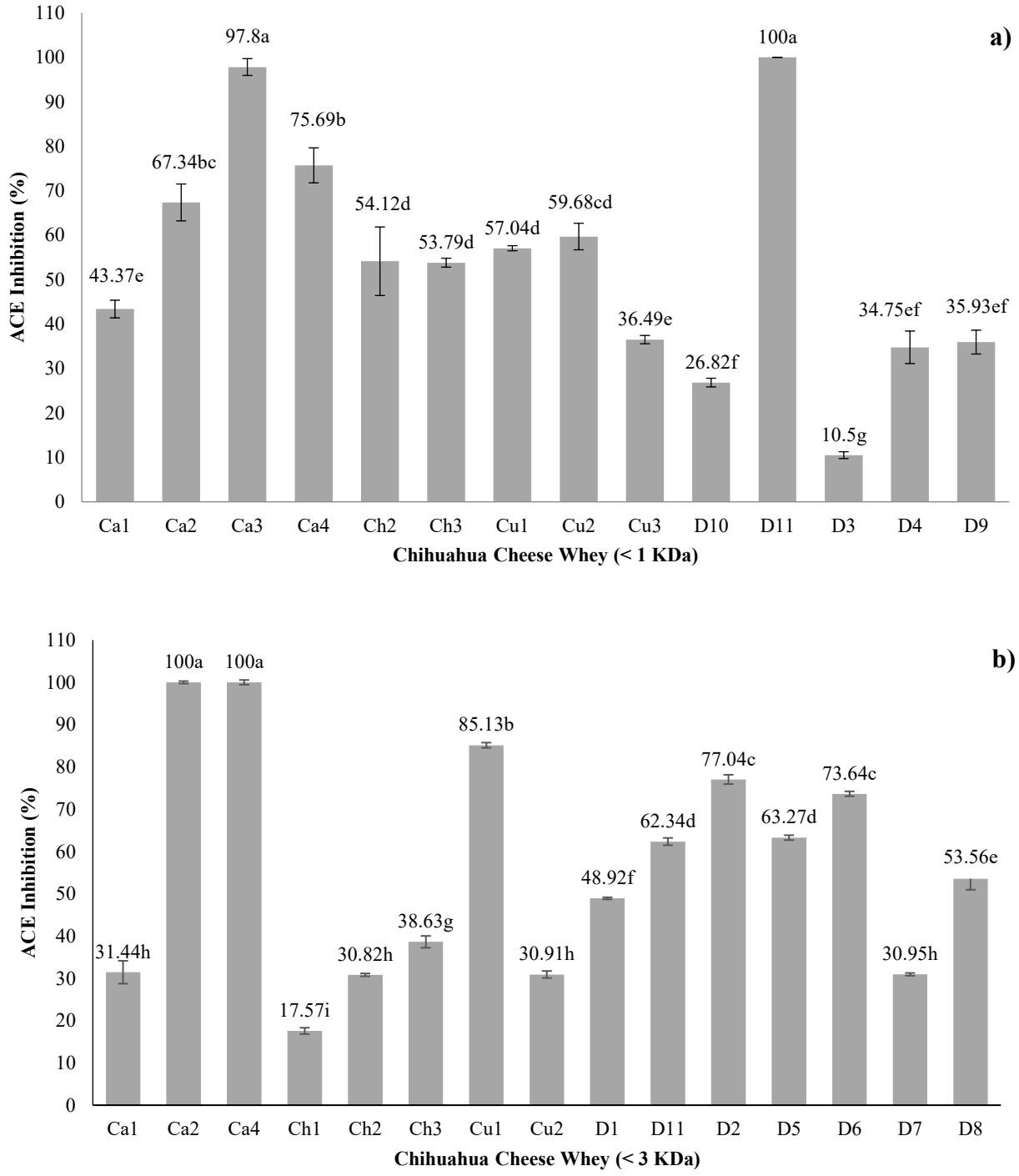


**Figure 2.** Percentage of Angiotensin I-converting enzyme (ACE)-inhibitory activity of Chihuahua cheese whey fractions: a < 1 KDa y b < 3 KDa. (Ca=Camargo; Ch= Chihuahua; Cu= Cuauhtemoc; and D=Delicias).
**Figura 2.** Porcentaje de actividad inhibidora de la enzima convertidora de angiotensina I (ACE) de fracciones de suero de queso Chihuahua: a < 1 KDa y b < 3 KDa. (Ca=Camargo; Ch= Chihuahua; Cu= Cuauhtémoc; and D=Delicias).

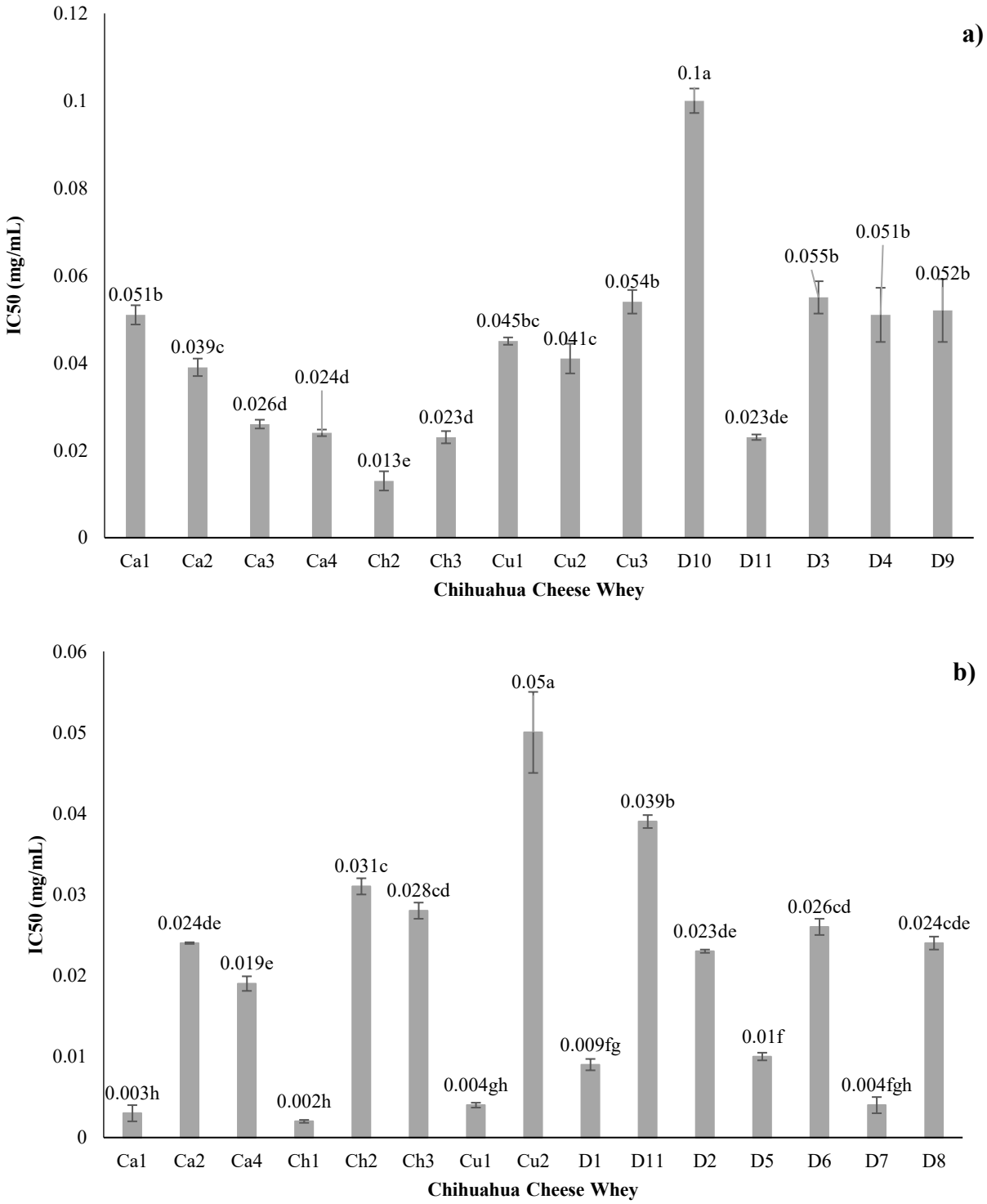


**Figure 2.** Angiotensin I-converting enzyme (ACE)-inhibitory activity of Chihuahua cheese whey fractions: a < 1 KDa y b < 3 KDa. (Ca=Camargo; Ch= Chihuahua; Cu= Cuauhtémoc; and D=Delicias).
**Figura 2.** Actividad inhibidora de la enzima convertidora de angiotensina I (ACE) de fracciones de suero de queso Chihuahua: a < 1 KDa y b < 3 KDa. (Ca=Camargo; Ch= Chihuahua; Cu= Cuauhtémoc; and D=Delicias).

Furthermore, the enzymes used during cheese processing will define the degree of hydrolysis in the whey(Shu et al., 2018)observed that the highest degree of hydrolysis and ACE inhibition was obtained in the alkaline

protease hydrolysates when compared with trypsin, bromelain and papain. In addition, the ACE inhibitory activity of hydrolysates from goat milk was higher than the ones from cow milk. Also, it has been reported that different strains of lactic acid bacteria can release peptides of various sizes and biological activity(Torres-Llanez et al., 2011). Boutrou et al., (1998)reported that hydrolysis of milk proteins results in peptides with different functions, positive or negative.

## 4. Conclusions

There was a high variability in the degree of proteolysis and ACE inhibition activity of whey. However, all samples inhibited ACE. It may imply that any whey from Chihuahua cheese manufacture has ACE inhibitory activity and may be used as a functional ingredient or food.

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**Contribution of the authors in the development of the work**

Flores-Mancha, Martha Azucena: She drafted the manuscript.
Paredes-Montoya,Pedro: He made the experimental work.
Renteria-Montertubio, Ana Luisa: She analyzed data.
Chavez-Martinez, America: She was the leader of the project, planned the research and she contributed with the revision of the manuscript.

**Interest conflict**

There is no conflict of interest among the authors

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