Antiulcerogenic Activity of Fraction and Hydrolysate Obtained from Whey Protein Concentrate

Atividade Antiulcerogênica de Hidrolisados de Soro de Leite e Frações Obtidos de Concentrado Protéico de Soro de Leite

AUTORES
AUTHORS

✉ **Maria Teresa Bertoldo PACHECO**[1*]
Érica A. BIGHETTI[2]
Márcia ANTÔNIO[2]
João Ernesto de CARVALHO[2]
Ana POSSENTI[2]
Valdemiro Carlos SGARBIERI[3]

[1*]Centro de Química de Alimentos e Nutrição Aplicada,
Instituto de Tecnologia de Alimentos (ITAL),
Av. Brasil, 2880, Campinas, SP, Brasil, Box 139, CEP 13070-178,
[2]Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas,
Universidade Estadual de Campinas, Caixa Postal 6171,
CEP 13081 970, Campinas, SP, Brasil
[3]Departamento de Alimentos e Nutrição,
Faculdade de Engenharia de Alimentos,
UNICAMP, Campinas, SP, Brasil.
*e-mail: mtb@ital.sp.gov.br

SUMMARY

The purpose of this research was to test the ability of a whey protein concentrate hydrolysate (WPH) obtained by treatment with pancreatin and its fraction of low molecular weight (WPHF, peptides 1kDa) to inhibit gastric mucosa ulcerative lesions caused by oral administration to rats of absolute ethanol. The WPH and WPHF were administrated in single and double dosis and compared to a whey protein concentrate (WPC). It was investigated the metabolic routes of cytoprotection by alkylation of sulfhydryl compounds, glutathione inhibition, nitric oxide and prostaglandin synthesis for WPH and WPHF. Acute administration (single dosis) of WPH resulted in 65.5% inhibition of the ulcerative lesion index (ULI), and 78.3% inhibition was obtained with repetitive dosis. For the whey protein hydrolysate fraction (WPHF) inhibition of ULI was 69.3% for single dosis and 64.6% for double dosis. Alkylation of SH-compounds by a subcutaneous injection of N-ethylmaleimide (NEM) dropped the protective effect of WPH and WPHF to 36.6% and 35.3%, respectively. Intraperitoneal injection of butathionine sulfoximine (BSO), which inhibit glutathione synthesis, dropped to a lesser extent the ULI inhibition of WPH and WPHF 65.74 and 60.43%, respectively. Indomethacin (10mg/ Kg body weight), which is a potent inhibitor of endogenous prostaglandin synthesis, reduced the protective effect of WPHF to 11%. These results may suggest that WPH and WPHF have antiulcerogenic activity against ethanol damage to gastric mucosa, which in part depends on sulfhydryl compounds present in the WPH and WPHF. The antiulcerogenic protective effect of peptides presented in WPHF may be exerted, especially through stimulation of endogenous prostaglandin synthesis.

RESUMO

A proposta deste trabalho foi testar a habilidade do hidrolisado de proteínas de soro de leite (WPH), obtido pelo tratamento com pancreatina e sua fração de baixo peso molecular (WPHF, peptídeos 1kDa) em inibir a mucosa gástrica de lesões ulcerativas causadas pela administração oral de etanol absoluto. O WPH e WPHF foram administrados em dose simples e dose dupla e comparados ao concentrado protéico de proteína (WPC). Foram investigadas as rotas metabólicas de citoproteção por aquilação dos compostos sulfidrilas, inibição da síntese de glutationa e prostaglandinas para o WPH e WPHF. A administração aguda (dose simples) do WPH resultou em 65% de inibição do índice de lesão ulcerativa (ULI), e 78,3% de inibição foi obtido em dose repetitiva. Para a fração do hidrolisado de proteínas do soro (WPHF) a inibição do ILU foi de 69,3% para a dose simples e 64,6% para dose dupla. A alquilação dos compostos SH por uma injeção subcutânea se N-etil maleimida (NEM) dobrou o efeito protetor de butationa sulfoximida (BSO), o qual inibe a síntese de glutationa, estendeu a inibição do ILU do WPH e WPHF de 65,74% e 60,43%, respectivamente. A indometacina (10mg/Kg de peso corporal), um potente inibidor da síntese de prostaglandinas endógenas, reduziu o efeito protetor do WPHF para 11%. Estes resultados podem sugerir que o WPH e WPHF têm atividade antiulcerogênica contra os efeitos deletérios do etanol na mucosa gástrica, o qual em parte depende dos compostos sulfidrilas presentes no WPH e WPHF. O efeito protetor antiulcerogênico dos peptídeos presentes no WPHF parece estar relacionado, especialmente, ao estímulo da síntese de prostaglandinas endógenas.

BRAZILIAN JOURNAL OF FOOD TECHNOLOGY

PACHECO, M. T. B.; BIGHETTI, É. A.;
ANTÔNIO, M.; De CARVALHO, J. E.;
POSSENTI, A. & SGARBIERI, V. C.

Antiulcerogenic Activity of Fraction
and Hydrolysate Obtained from
Whey Protein Concentrate

1. INTRODUCTION

Ethanol is an irritant agent to the gastric mucosa, which is capable of destroying the mucus and bicarbonate layer protect the mucosa against gastric acid and other chemical aggressors. Ethanol acts blocking the cytoprotection by precipitating proteins, originating free radicals and decreasing concentration of sulfhydryl compounds in the mucosa cells. Investigations in this area has found that extracts and vegetable oils are able to protect the gastric mucosa of the deleterious effect of ethanol, by stimulating the synthesis of glutathione (GSH) in the cells of the gastric epithelium. It was attributed to different mechanisms mediated by glutathione; the first one would be through the synthesis and increased concentration of glutathione in the cells and subsequently GSH mediating the formation of different anti-inflammatory prostaglandins. The GSH acts as cofator in some steps in the synthesis of prostaglandin acting in the conversion of the PG_2 to PGH_2 and the subsequent conversion to PGE_2. The prostaglandin synthetase enzyme is inable to synthesize PgE_2 in the depletion of glutathione [1-3].

Bovine milk whey proteins have been pointed out as nutrient possessing functional properties capable of modulating some physiological activities in the animal organism. Ability in increasing the immune response has been attributed to the milk whey protein concentrate (WPC), which participate in the combat of inflammatory infections, antiulcerogenic activity and carcinogenic processes. Attention is now dedicated to whey proteins fractionation in order to investigate potential benefits in human health improvement [4-8]. The isolated -lactalbumin has shown about four fold higher protection to ethanol induced stomach lesion in rat than a whey protein isolate (WPI), which contained 25% -lactalbumin. Pretreatment with indomethacin (10 mg/kg bw, sub cutaneous injection) significantly reduced the protective effect of this fraction. As indomethacin is a potent inhibitor of endogenous prostaglandin synthesis, the authors suggested that the antiulcerogenic protective effect of alpha-lactalbumin might be exerted, in part, through stimulation of endogenous prostaglandin synthesis [9]

Although milk whey proteins presents high digestibility and are quickly absorbed it is known that protein hydrolysates containing peptides of small sizes; di and tri-peptides, are absorbed faster relative to proteins as free amino acids [10]. It's also known that hydrolysates in the form of di and tri-peptides are more stable to sterilization and storage preserving their stability, increasing the solubility without affecting the osmolarity in the intestinal lumen [11,12].

Based on the evidence that a whey protein concentrate exert a protective action against the development of gastric mucosa ulcerative lesions, we decided to investigate a possible inhibitory effect of hydrolysates of whey proteins obtained by treatment with pancreatin and its low molecular weight peptides fraction (MW 1kDa), and exploring possible mechanisms of protection.

Table 1 Amino acid profiles of whey protein concentrate (WPC) and its hydrolysate (WPH) produced by pancreatin and low molecular weight peptides fraction (WPHF).

Amino acids (g/100g prot)	WPC	WPH	WPHF
Asp	10.50	10.71	10.57
Thr	6.88	7.17	6.48
Ser	5.53	5.48	5.14
Glu	17.81	18.36	18.77
Pro	5.97	5.95	5.64
Gly	1.79	1.76	1.75
Ala	4.75	4.71	5.32
Cys	2.45	2.08	2.07
Val	5.42	5.07	5.70
Met	2.46	1.98	2.11
Ile	5.69	5.31	6.80
Leu	10.55	10.53	11.49
Tyr	3.14	2.98	2.48
Phe	3.44	3.23	3.70
Lys	9.97	9.82	8.64
His	2.54	1.63	2.49
Arg	1.56	1.86	0.90

*Try (Tryptophan) destroyed by acid hydrolysis, previous to analysis.

2. MATERIALS AND METHODS

2.1 Whey Protein Concentrate (WPC) and Hydrolysate (WPH)

The whey protein concentrate utilized for manufacture of hydrolysate was produced in pilot plant from defatted and pasteurized milk, according procedure describe by BORGES et al. [13]. Hydrolysis was conducted in a pH-*stat* (model 716, Methron, Les Uleis, France) at 10% substrate concentration (w/v) and degree of hydrolysis (DH) was 20%. WPC was hydrolyzed with the enzyme system porcine pancreatin (Sigma P-1750) using temperature of 37 °C and pH 7.5. Degree of hydrolysis, monitored by the pH-stat method was calculated as recommended by ADLER-NISSEN [14]. At the end of the incubation period the enzymes were inactivated by heating at 85 °C for 15 min, following freezing and freeze-drying.

2.2 Whey Protein Hydrolysate Fraction (WPHF 1kDa)

WPH 5% in water (w/v) was submitted to tangential flow filtration using Prep/Scale - TFF cartridges $1ft^2$ (MW cut-off 1kDa). The recovered permeate with water flow was freeze dried and stored at 5^0C for tests.

PACHECO, M. T. B.; BIGHETTI, É. A.;
ANTÔNIO, M.; De CARVALHO, J. E.;
POSSENTI, A. & SGARBIERI, V. C.

**Antiulcerogenic Activity of Fraction
and Hydrolysate Obtained from
Whey Protein Concentrate**

2.3 Amino acids determination

Amino acids were determined in HPLC autoanalyzer (Dionex Dx-300) by separation in a cation exchange resin and post-column ninhydrin reaction. Quantification of the amino acids was based on an amino acid standard mixture (Pierce Kit 22).

2.4 Distribution of peptides and amino acids in the whole hydrolysate and 1kDa fraction

Quantification of peptides from proteolysis by molecular size distribution and free amino acids was possible using high-resolution molecular exclusion chromatography (SE-HPLC). Column Polish-(2-hidroxietilaspartamide)-silica (PHEA) 250 x 9.4, 5 m and 200A (PolylC, Columbia, MD) was used. The sample was dissolved 1% (w/v) in formic acid 0.05M and analyzed with a flow rate of 0.5mL/min, under isocratic conditions at room temperature for 35 minutes. The peaks were detected in the wavelengths of 230 nm. The free peptides and amino acids were quantified by the fast method of area corrected fraction by comparison with a standard curve [15].

2.5 Drugs and reagents

Ethanol, sodium chloride, carbenoxolone (C4790), N- -L-arginine methyl esther (N5751), N-ethylmaleimide (NEM-E1271), indomethacin (I7378) and butathionine sulfoximine (BSO-B2515) were all Sigma reagent.

2.6 Animals and diets

The animals used were adult male rats of the Wistar strain weighing from 300-400g, acquired from the experimental animal center (CEMIB) of the University of Campinas, Campinas, São Paulo, Brazil. Prior to the experiment, the animals were maintained for at least seven days at the experimental laboratory at 20ºC and alternating cycles of 12 h light and 12 h dark, receiving commercial ration and water *ad libitum.*

Ulcer induction. For induction of gastric mucosa ulcerative lesions, seven rats were used in each treatment. The experimental protocol was approved by the Ethics Committee for Animal Research of the University of Campinas in agreement with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation (COBEA). The model was according to Robert [16], using saline solution (sodium cloride 0,9%) as negative control, carbenoxolone as positive control, WPC, WPH e WPHF as the testing sample. Absolute ethanol was used as the ulcerogenic agent. Details of the experiments are given in Figure1.
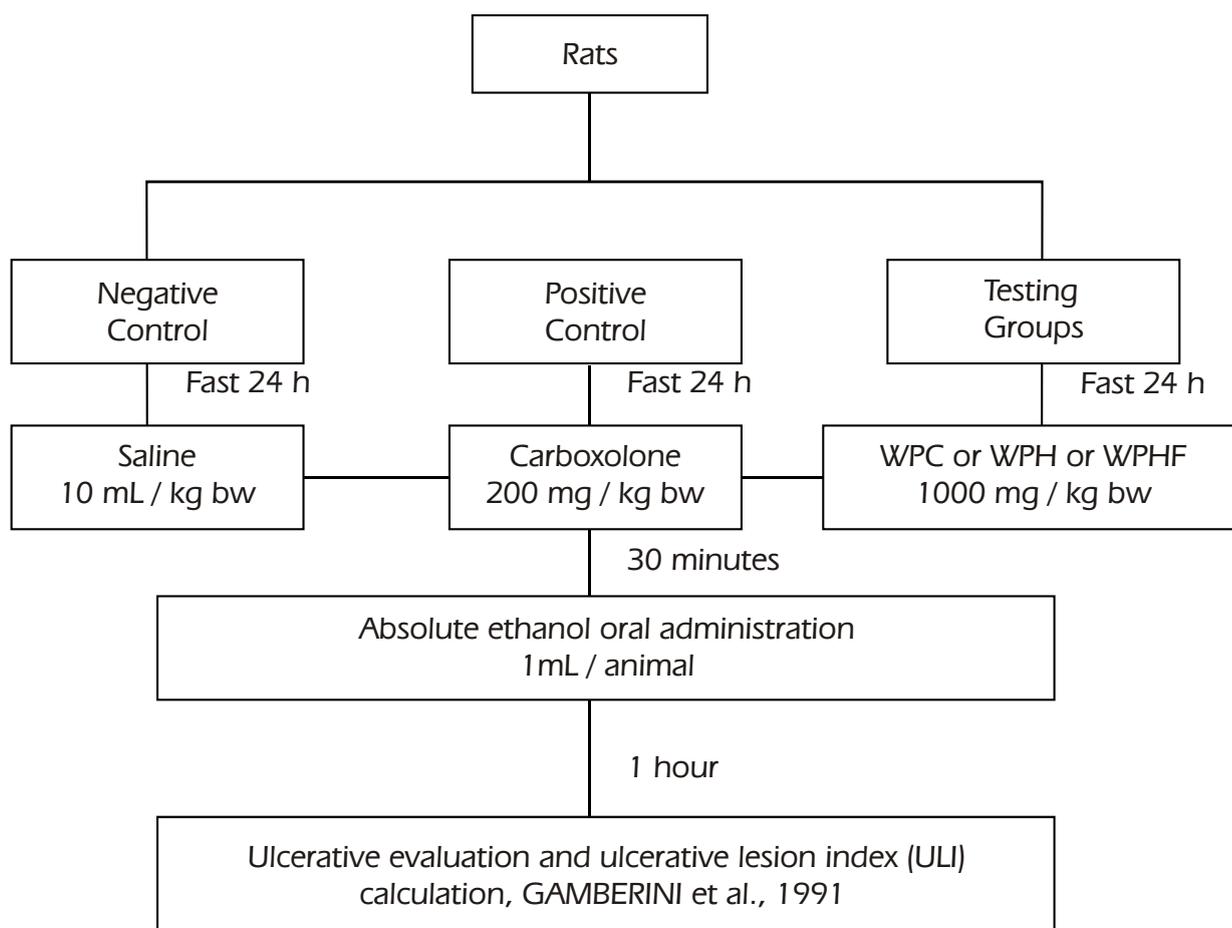
Using the same model, two different experiments were performed, i.e., acute and repetitive oral administration of the WPH and WPHF. For the acute (single dosis) experiment the protocol was exactly as illustrated in Figure 1. For the repetitive dosis experiment, the animals were fasted 24 h prior to receiving the first treatment, as in the single dosis experiment. The rats were then fed commercial diet for 8 h followed by 16 h fasting when the second treatment was given. Thirty minutes after the second treatment all the rats received orally 1 mL absolute ethanol/animal. Elapsed 1 hour the animals were sacrificed by cervical dislocation, their stomach cut open, along the line of greater curvature, washed with saline for ulcerative lesions detection.

Index of ulcerative lesions (ULI) The index of ulcerative lesions was evaluated by summing up the number of lesions, considering their size, and taking also into account other parameters like occurrence of hemorrhage, folding loss and discoloration, according to methodology of GAMBERINI et al

Table 2 Effect of a single and double dosis of a WPC, WPH, WPHF and carbenoxolone in the protection of rat stomach ulcerative lesions caused by oral administration of absolute ethanol in rats.

Inductive Model	Treatment	ULI[1] (mean $\pm$ SEM)	Inhibition[2] of ULI (%)
	WPC [3]	58.83 $\pm$ 19.00*	38.2
Ethanol	WPH [4]	32.8 $\pm$ 4.92 ***	65.5
Single dose*	WPHF [5]	29.20 $\pm$ 11.56***	69.3
	Carbenoxolone	13.73 $\pm$ 4.13***	85.5
	Saline	95.20 $\pm$ 12.14	
Ethanol	WPC [3]	30.50 $\pm$ 9.95***	69.4
Double dosis**	WPH [4]	21.6 $\pm$ 4.7***	78.3
	WPHF [5]	35.28 $\pm$ 11.62***	64.6
	Carbenoxolone	15.09 $\pm$ 4.30***	84.8
	Saline	99.70 $\pm$ 14.44	

[1]Ulcerative lesions index, [2]percentage inhibition of ulcerative lesions, [3]whey protein concentrate, [4]whey protein hydrolysate, [5]whey protein hydrolysate fraction (MW < 1kDa). * ANOVA $F_{(2,14)}$ = 87.5 p< 0.001, Duncan test ***p<0.001, *p< 0.05 referred to saline.

Braz. J. Food Technol., III JIPCA, janeiro, 2006

17

PACHECO, M. T. B.; BIGHETTI, É. A.;
ANTÔNIO, M.; De CARVALHO, J. E.;
POSSENTI, A. & SGARBIERI, V. C.

Antiulcerogenic Activity of Fraction
and Hydrolysate Obtained from
Whey Protein Concentrate

BRAZILIAN JOURNAL
OF FOOD TECHNOLOGY

```
                        ┌─────────────┐
                        │    Rats     │
                        └─────────────┘
        ┌──────────────────────┼──────────────────────┐
┌───────────────┐      ┌───────────────┐      ┌───────────────┐
│   Negative    │      │   Positive    │      │    Testing    │
│   Control     │      │   Control     │      │    Groups     │
└───────────────┘      └───────────────┘      └───────────────┘
        Fast 24 h              Fast 24 h              Fast 24 h
┌───────────────┐      ┌───────────────┐      ┌───────────────────┐
│    Saline     │      │  Carboxolone  │      │ WPC or WPH or WPHF │
│  10 mL / kg bw│      │ 200 mg / kg bw│      │  1000 mg / kg bw   │
└───────────────┘      └───────────────┘      └───────────────────┘
                          30 minutes
        ┌─────────────────────────────────────────┐
        │    Absolute ethanol oral administration  │
        │              1mL / animal                │
        └─────────────────────────────────────────┘
                           1 hour
        ┌─────────────────────────────────────────┐
        │ Ulcerative evaluation and ulcerative     │
        │ lesion index (ULI) calculation,          │
        │ GAMBERINI et al., 1991                   │
        └─────────────────────────────────────────┘
```

Figura 1 Experimental protocol for the induction of stomach ulcer in rats by absolute ethanol oral administration.

[17] with the following criteria (where n* = number of ulcers found):

Loss of normal morphology, 1point/ Mucosa discoloration, 1point/ Mucosal edema, 1point, Hemorrhages, 1 point/ Petechial points (until 9), 2 points/ Petechial point (10 or more), 3points/ Ulcers (up to 1 mm), n* x 2 points/ Ulcers (> 1 mm or more), n* x 3 points/ Perforated ulcers, n* x 4 points

Percent Ulcerative Lesion Inhibition (ULI %). The percentage inhibition of ulcerative lesions was calculated by comparing the lesions in the testing sample treatment with the negative control (saline) treatment. The following expression was used [17]:

ULI(%) = average negative control average test sample / average negative control x 100

Estimation of the effective dose. The effective dosis (ED_{50}) was determined by using the single dosis protocol and increasing dosis of 250, 500, 750 and 1000 mg/kg body weight (bw) of WPC were administered orally to the rats after 24 hours of fasting. The estimed ED_{50} was determined by use a linear regression equation. The dose of 1000mg/kgbw was standardized for all treatments [8].

Treatment with N-ethylmaleimide (NEM). Participation of sulfhydryl (SH) compounds on the protection of stomach ulcerative lesions by ethanol were performed the repetitive treatments (double dosis). Groups of 5 male Wistar rats (300-400 g) were submitted to fasting for 24 h before the first treatment, using saline solution as negative control, WPH and WPHF as the testing sample, as for the single dosis. After the first treatment, the animals were fed a commercial ration for 8 hours, followed by 16 hours fasting. Then three groups (5 rats) received subcutaneous injection (10 mg/kg bw) of N-ethylmaleimide (NEM). Thirty min after NEM injection one group (5 rats) received orally 10 mL/kg bw of saline (negative control), the other two groups received a 1000mg/kg body weigh of either WPH or WPHF. Thirty min after treatments all the rats were orally administered with 1 mL/animal of absolute ethanol (ulcerogenic agent). One hour after ethanol administration the animals were sacrificed by cervical dislocation, the stomachs were removed, cut open along the line of greater curvature, washed with saline and the index of ulcerative lesions (ULI) determined [17].

Treatment with butathionine sulfoximine (BSO). To evaluate the importance of glutathione in the cytoprotection mechanism by WPH or WPHF, a protocol similar to NEM was applied [18]. Intraperitoneal injection (4 mmol/kg bw) of butathionine sulfoximine (BSO) was given for all treatments.

BRAZILIAN JOURNAL OF FOOD TECHNOLOGY

PACHECO, M. T. B.; BIGHETTI, É. A.;
ANTÔNIO, M.; De CARVALHO, J. E.;
POSSENTI, A. & SGARBIERI, V. C.

**Antiulcerogenic Activity of Fraction
and Hydrolysate Obtained from
Whey Protein Concentrate**

After four hours after BSO injection was given a second dosis with test treatment (WPH, WPHF). The negative control received saline (10 mL/kg bw) again. Thirty minutes after this second treatment, the ethanol (1mg/animal) was given orally to all the rats, and 1 hour later the animals were sacrified for stomach ULI determination [17].

Treatment with indomethacin (IND). The participation of endogenous prostaglandin in the protection mechanism by WPH or WPHF was examined by using the method described by MARTIN et al. [19] in repetitive dosis. Thirty minutes prior to the administration of the second treatments (WPH, WPHF and saline) 10 mg/kg body weight of indomethacin was given subcutaneously. After 1 hour the rats were then treated by ethanol model (1 mL/animal) and 1 hour later the animals were sacrified by cervical dislocation, the stomachs removed, cut open, and ulcerative lesion index (ULI) determined.

Treatment with N - L-arginine methyl esther (L-NAME)

To determinate the role of nitric oxide in the cytoprotection the animals received a subcutaneous injection (5mg/kgbw) of L-NAME (N - L-arginine methyl ester), in double dosis treatment, conform prior experimental protocol. After thirty minutes, all animal groups received orally the respective test treatment (WPH or WPHF) and saline. After an hour, the animals were orally administrated with absolute ethanol (1mL/animal). These animals were sacrified one hour later and their stomachs were removed and opened along the greater curvature. The lesions were evaluated and the ulcerative index lesion (ULI) was calculated [17].

Statistical analysis. Experimental results were submitted to analysis of variance (ANOVA). Differences among means were evaluated by the Duncan test at a minimum critical significant level of 5% probability (p 0.05).
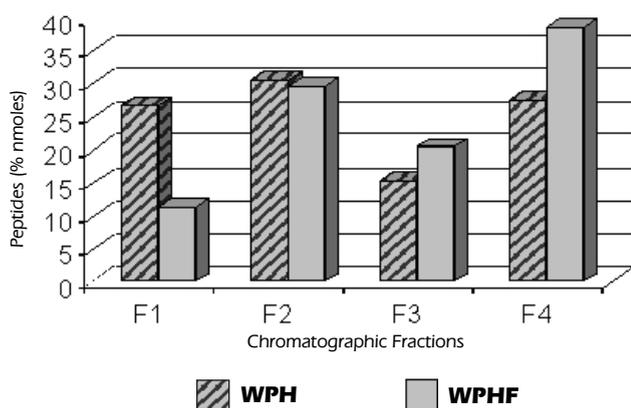
Figura 2 Distribution of peptides by molecular weight: WPH, Whey protein hydrolysate; WPHF, fraction < 1kDa; F1, peptides with more than 7 amino acids residues; F2, peptides with 4-7 amino acids residues; F3, peptides with 2-3 amino acids residues and F4, free amino acids.

3. RESULTS AND DISCUSSION

3.1 Amino acid profiles

Amino acids predominant in the WPC are aspartic acid, threonine, glutamic acid, leucine, and lysine (Table 1). The high content of branched chain amino acids, leucine particularly, has been emphasized in the literature as important for regeneration of tissues in multiple traumas and burns [20]. Another important characteristic of whey proteins is the low content aromatic amino acids. The 1:1 ratio of methionine to cysteine makes the WPC similar to human milk. Hydrolysis procedure did not modify the amino acid composition of WPH. However the ultrafiltration process to obtain the WPHF resulted in some amino acids increase such as Ile, Leu, Thr, Glu, Ala and Val. The sulphur amino acids were little altered (Cys + Met).

3.2 Composition and distribution of peptides and amino acids in a total hydrolysate and fraction low MW (1KDa)

The distribution of peptides in the WPH can be seen in Figure 2. The basic difference between WPH and WPHF consists in the prevalence in WPH of peptides with more than seven amino acids residues (F1) while in the WPHF predominate peptides with two and three amino acids residues (F3) and free amino acids (F4). Figure 2 shows those peptides with more than seven amino acids residues accounts for about 25% of the WPH composition and only 10% of WPHF composition (F1). Peptides with four to seven (F2) amino acids residues represent about 30% of the whole sample. 15% of WPH and 20% of WPHF composition are formed of peptides with two or three amino acids residues, approximately. The free amino acids account for 25% and 37% the WPH and WPHF, respectively.

3.3 Evaluation of the Antiulcerogenic Activity

In the acute model of induced ulcer by ethanol the WPC was used as reference and presented an index of ulcerative lesion (ULI) superior to the whey protein hydrolysate (WPH) and the whey protein hydrolysate fraction (WPHF) in the dose of 1000mg/kg bw (Table 2). The results of ULI presented significance difference (p< 0.001) referent to the negative control (saline treatment).

Results of double dose treatment of hydrolysates showed similar statistics, indicating of protective effect of the samples, which was inferior to the one presented by carbenoxolone (positive control). The WPC was more effective in double dosis inhibiting about 70% the ULI, while carbenoxolone inhibited 85% for the ethanol model and double dose. Theses results are similar to the ones reported by LOGUERCIO et al. [2]. The whey protein hydrolysate fraction (MW < 1kDa) was already efficient in the first dose and conserved or extended its protective effect for repetitive dosis in the ethanol model. All treatment presented significant difference (p<0.001), based on saline values (negative control).

3.4 Evaluation of the cytoprotection mechanisms

This study was based on the hypothesis that the

BRAZILIAN JOURNAL OF FOOD TECHNOLOGY

PACHECO, M. T. B.; BIGHETTI, É. A.;
ANTÔNIO, M.; De CARVALHO, J. E.;
POSSENTI, A. & SGARBIERI, V. C.

Antiulcerogenic Activity of Fraction
and Hydrolysate Obtained from
Whey Protein Concentrate

protective effect against ulceration of milk whey protein could be attributed to the high sulphur amino acids content as well as to several sequences glutamic acid-cystine found in these proteins and considered essential for efficient glutathione biosynthesis. In the small MW fraction (MW< 1kDa) with peptides of fewer amino acids residues it is expected that more efficient absorption and biological utilization than for the intact protein (WPC) or whole hydrolysate (WPH). Some of these small peptides with the glutamic acid-cystine sequences would serve as preferred substrate for glutathione biosynthesis.

3.5 Alkylation of total sulfhydryl groups with N-ethylmaleimide (NEM)

N-ethylmaleimide (NEM) is used to temporarily block "in vivo" the function of all active SH groups in the body. The treatment consists of NEM prior to the treatment with saline and with the experimental treatments. Table 3 show the effects of blocking different metabolic routs with specific blocking agents on the protection of gastric mucosa by whey protein hydrolysate (WPC) and low MW hydrolysate fraction (WPHF) compared with saline plus specific blocking agent as negative control.

When total SH groups were blocked with NEM the ULI remained quite high and differed from the negative control ($p < 0.01$) indicant that treatment with NEM interferes with WPH and WPHF efficiency in protecting the gastric mucosa of ethanol ulceration.

The interference of about 36% in both WPH and WPHF mucosa protection suggest that total active sulfhydryl compounds play an important role on mucosa protection by whey protein hydrolysates. The sulphur amino acids content of whey protein (5.0g/100g protein) is considerably higher than most dietary proteins. The proteins -lactoglobulin, bovine serum albumin, -globulins, lactoferrin and lysozyme are rich source of sulphur amino acids [22].

According to SAZBO [21] and TAUCKECHI [22], both sulphur amino acids and glutathione seem to inhibit free radicals and prevent an increase in vases permeability in the mucosa promoted by the action by ethanol.

3.6 Participation of glutathione

Treating the rats with intraperitoneal injection of butathionine sulfoximine (BSO) specifically blocks the glutathione biosynthesis. As shown in Table 2 this treatment did not interfere severely with the efficacy of WPH and WPHF to protect the stomach mucosa. Compared with negative control the mucosa protection continued highly significant ($p < 0.001$) for both WPH and WPHF, corresponding to a high percentage (60 - 65%) of inhibition the ULI, even after treatment with BSO. These results suggests that glutathione synthesis was relatively unimportant in these experiments, in spite of demonstration [23] that undenatured whey protein concentrate (WPC) promoted an important stimulus to glutathione synthesis in various tissues, including gastric mucosa. ROSANELI [8] demonstrated that WPC offered a high level of protection against ulcerative lesions caused by ethanol, suggesting that SH-compounds and glutathione played an important role as protecting agents.

3.7 Nitric oxide cycle

Nitric oxide is an endogenous substance that enhances the mechanisms of gastric cytoprotection increasing mucosal blood flow and inhibiting the release of oxygenated metabolites and proteases from leucocytes in the gastric microcirculation. The nitric oxide also modulates the secretion of some cellular growth factors, mainly the epidermal growth factor [24]. The participation of nitric oxide was evaluated on ethanol induced ulcer model with previous administration of N -L- argininemethyl ester (L-NAME), a NO-synthetase inhibitor. Under these conditions, the WPH and WPHF maintained the antiulcerogenic activity suggesting that nitric oxide could not be involved in the antiulcerogenic activity of these treatments (Table 2 and 3).

After treatment with L-NAME the protecting capacity to the mucosa of WPH and WPHF remained very high and significant in relation the negative control ($p < 0.001$). The protection offered after of 75 - 76% compared to the negative control.

3.8 Participation of prostaglandin (PGE$_2$)

Prostaglandins protect the gastric mucosa through many mechanisms, such as decrease of acid secretion, bicarbonate and mucus production as well increasing the

Table 3 Effect of pretreatment with various blocking agents on the protective effect of WPH and WPHF on % inhibition of (index of ulcerative lesions) ULI using double dosis ethanol model in rats.

Treatment	ULI (mean±SEM)	ULI INIBITION (%)
WPH+NEM [a]	56.8 ± 15.4**	36.61
WPHF+NEM	58.0 ± 22.1**	35.27
SALINE+NEM	89.6 ± 11.7	
WPH+L-NAME [b]	23.6 ± 7.8***	76.45
WPHF+L-NAME	25.0 ± 8.5***	75.05
SALINE+L-NAME	100.2 ± 17.5	
WPH+BSO [c]	32.2 ± 14.0***	65.74
WPHF+BSO	37.2 ± 16.9***	60.43
SALINE+BSO	94.0 ± 15.4	
WPH+IND [d]	32.8 ± 7.3**	51.76
WPHF+IND	60.2 ± 9.1[ns]	11.47
SALINE+IND	68.0 ± 14.1	

(a) ANOVA $F_{(3.16)}$ = 9.2 $p < 0.01$, Duncan's test **$p < 0.01$; (b) ANOVA $F_{(3.16)}$ = 24.0 $p < 0.01$, Duncan's test ***$p < 0.001$; (c) ANOVA $F_{(3.20)}$ = 93.5 $p < 0.001$, Duncan's test ***$p < 0.001$; (d) ANOVA $F_{(3.16)}$ = 76.4 **$p < 0.01$, Duncan's test **$p < 0.01$. ns = non significant. Statistical was perfomed for all different blocks of experiments (a,b,c,d). Saline plus the specific blocking agent was considered as negative control. NEM, N-ethymaleimide; L-NAME, N -L-argininemethyl esther; BSO, butathionine sulfoximine; IND, indomethacin.

Braz. J. Food Technol., III JIPCA, janeiro, 2006

20

PACHECO, M. T. B.; BIGHETTI, É. A.;
ANTÔNIO, M.; De CARVALHO, J. E.;
POSSENTI, A. & SGARBIERI, V. C.

**Antiulcerogenic Activity of Fraction
and Hydrolysate Obtained from
Whey Protein Concentrate**

mucosal blood flow [25]. The indomethacin is a drug that inhibits the ciclooxogenase enzyme involved in prostaglandin synthesis. After treatment with indomethacin the antiulcerogenic activity was maintained for WPH, suggesting that these sample there was no interference of prostaglandins on gastric cytoprotection. However the cytoprotection activity to the gastric mucosa of WPH was drastically reduced to a non significant leve (11.5%) only of prostaglandins production suggesting that the gastric cytoprotection of WPHF is highly dependent on prostaglandin action.

The behavior observed for WPH was quite different from that of WPHF. Blocking the synthesis of PG_2 by indomethacin administration did not eliminate WPH mucosa protection remaining significant (p< 0.01) relative to the negative control (52% protection).

4. CONCLUSION

In this work it was possible to develop and to standardize the conditions for attainment of two products with active physiological properties: hydrolysate of whey proteins concentrate (WPH) with high hydrolysis degree (20%) and whey proteins fractioned hydrolysate (WPHF) contend peptides with molecular weight below of 1kDa. WPC pancreatin hydrolysate (20% DH) was separated into 4 peptides fractions (F1-F4). F1 contained peptides with more than seven amino acids residues. F2 and F3 contained peptides in the range of 2 7 amino acids residues and F4 contained only free amino acids. WPC, WPH and peptides fraction smaller than 1kDa showed gastric antiulcerogenic activity on rats treated with ethanol. Inhibition of ulcerative lesions raged from 38.2% (WPC) to 69.3% (WPHF), compared to carboxolona (85.5% positive control) in single dosis experiments. For double dosis treatments inhibition ranged from 64.6% (WPHF) to 78.3% (WPH), compared to carboxolona (84.8%). The most effective route of protection seemed to be gastric cytoprotection by active sulfhydryl compounds and stimulation of prostaglandin production.

5. REFERENCES

1. EL-DAKHAKHNY M, BARAKAT M, EL-HALIM A, ALY SM. Effects of Nigella sativa on gastric secretion and etanol induced ulcer in rats. J Ethopharmacol, 72:299-304, 2000

2. LOGUERCIO LC, TARANTO D, BENEDUCE F. Glutathione prevents ethanol induced gastric mucosal damage and depletion of sulfhydryl compounds in humans. Gut, 34: 161-165, 1993.

3. HIROISHI H, SHIMADA T, IVEY KJ, TERANO A. Role of anti-oxidant defenses against ethanol-induced damage in cultures of rat gastric epithelial cells. J. Pharm Exp Therapeutic, 289:103-109, 1999.

4. KENNEDY RS, KNOK GP, BOUNOUS G, BARUCHEL S, LEE TDG. The use of a whey protein concentrate in the treatment of patients with metastatic carcinoma: phase I-II clinical study. Anticancer Res, 15: 2643-2650, 1995.

5. ANDERSON ME. Glutathione: an overiew of biosynthesys and modulation. Chemico-Biological Interact, 111-112:1-14, 1998.

6. BARUCHEL S, VIAU G. In vitro selective modulation of cellular glutathione by a humanized native milk protein isolate in normal cells and rat mammary carcinoma model. Anticancer Research, 16: 1095-1100, 1996.

7. MCINTOSH GH, ROYLE PJ, LE LEU PK. Whey protein as functional food ingredients? Int Dairy J 8: 425-434, 1998.

8. ROSANELI CF, BIGHETTI AE, ANTÔNIO MA, CARVALHO JE, SGARBIERI VC. Efficacy of a whey concentrate on the inibition of stomach ulcerative lesions caused by ethanol ingestion, J Medic Food 5: 221-227, 2002.

9. MATSUMOTO H, SHIMOKAWA Y, USHIDA Y, TOIDA T, HAYASAWA H. New biological function of bovine alpha-lactalbumin: protective effect against ethanol and stress-induced gastric mucosal injury in rats. Biosci Biotechnol Biochem 65: 1104-1111, 2001.

10. GRIMBLE RF, GRIMBLE G. Immunonutrition: Role of sulfur amino acids, related amino acids, and polyamines. J Nutr 14:605-610, 1998.

11. ADIBI AS. Intestinal transpot of dipeptides in man: relative importance of hydrolysis and intact absorption. J Clin Invest 50: 2266-2275, 1987.

12. CLEMENTE A. Enzymatic protein hydrolysates in human nutrition. Trends Food Sci Tecnol 11: 254-262, 2000.

13. BORGES PFZ, SGARBIERI VC, DIAS NGP, JACOBUCCI HB, PACHECO MTB, BALDINI V LS. Produção piloto de proteínas de leite bovino: Composição e valor nutritivo. Braz J Food Technol 52; 1-8, 2001.

14. ADLER-NISSEN J. Methods in Food proteins Hydrolysis, In Enzymatic Hydrolysis of Food Proteins. Elsevier Applied Science Publishers, London, pp: 110-131, 1986.

15. SILVESTRE MPC, HAMON M, YVON M. Analyses of protein hydrolysates. 1.Use of poly(2-hydroxyethil-aspartamide)-silica column in size-exclusion chromatography for the fractionation of casein hidrolisates. J Agric Food Chem 42: 2778-2782, 1994.

16. ROBERT A. Cytoprotection by prostaglandins. Gastroenterol 77:761-767, 1979.

17. GAMBERINI MT, SKORUPA LA, SOUCCAR C, LAPA AJ. Inhibition of gastric secretion by a water extract from Baccharis triptera, Mart. Mem. Ins. Oswaldo Cruz, 86: 137-139, 1991.

18. BARUCHEL S, VIAU G. In vitro selective modulation of cellular glutathione by a humanized native milk protein isolate in normal cells and rat mammary carcinoma model. Anticancer Research 16:1095-1100, 1996.

19. MARTIN MJ, LA CASA C, LASTRA C A, CABEZA J, VILLARGAS J, MOTILVA V. Antioxidant mechanisms envolved in gastroprotective effects of quercetin. Z Naturforsch 53: 82-88, 1998.

20. BRENNAN MF, CERRA F, DALY JM, FISCHER JE, MOLDAWE LL, SMITH RJ, VINNARS E, WANNEMACHER R,

Braz. J. Food Technol., III JIPCA, janeiro, 2006

21

PACHECO, M. T. B.; BIGHETTI, É. A.;
ANTÔNIO, M.; De CARVALHO, J. E.;
POSSENTI, A. & SGARBIERI, V. C.

**Antiulcerogenic Activity of Fraction
and Hydrolysate Obtained from
Whey Protein Concentrate**

YOUNG VR. *Branched-chain amino acids in stress and injury. J. Parent Ent Nutr* 10: 446-452, 1986.

21. SZABO S. *Sulfhydryl compounds may mediate gastric cytoprotection. Science* 214: 201-202, 1981.

22. TAKEUCHI K, OKADA M, NIIDA H, OKABE S. *Role of sulfhydryls in mucosal injury caused by ethanol: relation to microvascular permeability, gastric motility and cytoprotection. J Pharmacol Exp Ther* 248: 836-841, 1989.

23. BOUNOUS G, GOLD P. *The biological activity of undenatured dietary whey proteins: role of glutathione. Clin Inv Med* 14: 296-309, 1991.

24. ELLIOT SN, MSKNIGHT W, CIRINO G, WALLACE, JL. *A nitric oxide-releasing nonesteroidal anti-inflamatory drug accelerates gastric ulcer healing in rats. Gastroenterol,* 109: 524-530, 1995.

25. FELIX WL, MILLER JC, GUTH PH. *Dissociated effects of misoprostol on gastric acid secretion and mucosal blood flow. Dig Diseases Sci* 31. 86S-90S, 1986.

6. AKNOWLEDGMENTS

Braz. J. Food Technol., III JIPCA, janeiro, 2006

22