

Chromatographic Characterization of Products Isolated from Chrome Shavings

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ABSTRACT

Gel permeation chromatography and high performance liquid chromatography were employed for separation and chemical characterization of products isolated from chrome shavings. After enzymatic hydrolysis, the products isolated were peptides of higher molecular weight. Peptides of lower molecular weight and free aminoacids were the main products using sulfuric acid in chrome shavings solubilization. Glycine (17%), glutamic acid (10.6%), alanine (9.2%), and arginine (8.2%) were the principal amino acids found. Phenylalanine(1.8%) was the main aromatic amino acid, while tryptophane was completely absent.

Key words; Chrome shavings; enzyme; chromatography; protein, chromium

INTRODUCTION

Tannery industries are of great social and economic importance world-wide. In Brazil, about 600 industries produce approximately 22 million leather per year, approximately 10% of total world production (Kimura et al, 1996; Taylor et al., 1996). Tanneries use different types of processes to tan the leather, and tanning with chromium salts are of great environmental concern because of its by-products (Taylor et al,1991). Enzymatic processing of chrome shavings has been shown to be a viable treatment for complete solubilization. Various alkalinity-inducing agents such as magnesium oxide, calcium hydroxide, sodium carbonate, sodium hydroxide,etc.,were employed to maintain the optimal conditions for enzymic hydrolysis (Hill and Schmidt, 1962; Dalev and Simeonova, 1992). Carboxylates groups of protein form a strong and stable complex with chromium(III) in a mechanism of chelation and oxalation (O'Flaherty et al, 1965; Heidemann, 1997).

Due to this stable complex, the complete removal of chromium(III) from the soluble protein is a very difficult task, and high content of chromium is deleterious to the enzyme action (Kimura et al, 1996). The soluble protein may have commercial use as gelatines, animal feed supplement, peptides or amino acids production (Boushy et al, 1991; Tingda et al, 1992; Brown et al., 1994; Taylor et al., 1998). Chromium recovered after washing may be recycled by tanning industry or used in other industrial applications (Collivgnarelli and Barducci, 1991; Taylor et al., 1998).

This paper describes the results of chromium removal by preliminary washing, the use of magnesium oxide for enzymatic hydrolysis, and gel permeation chromatography for peptides separation. High performance liquid chromatography was employed for amino acids separation and characterization.

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MATERIALS AND METHODS

Apparatus

Atomic absorption spectrometer CG-AA-7000-ABC (AAS), with an air-acetylene burner was used for chromium determination after acidic digestion. UV-VIS-Beckman DU-70 spectrophotometer was used for peptides determination (Kimura et al, 1996). Thermostatic bath, Tecnal TE-184, temperature range of 0 to 99°C ± 1°C was used for chrome shavings hydrolysis. High performance liquid chromatography of amino acids separation was performed with HPLC-LDC-Analytical (Pickering Laboratories, Inc.) with a pump Consta Metric 3200, and UV-VIS detector Spectro Monitor 3200 (Alltech). The following columns were also used: Sodium cation exchange (8µm, 3 x 200 mm), (Pickering Laboratories, Inc.), and a pre-column (8 µm, 2 x 200 mm).

Reagents

Proteolytic enzyme **Alcalase 2.5 L DX**, supplied as a liquid, with activity of 2.5 (Anson unity) and optimal activity at pH 8.5-9.5 (Nova Nordisk S/A-Brazil); Biuret reagent (Bioclin-Quibasa); standard solution of protein mixture IV (12300-78000 Daltons-Merck); standard solution of 0.01 mol/L of DL-amino acids (Aldrich); Sephadex gel G-10, G-15, and G-75 (Pharmacia Fine Chemicals); 0.4% (m/v) solution of blue dextran (Sigma); 0.2% (m/v) solution of ninhydrin (Sigma) dissolved in acetone; 0.02% (m/v) solution of sodium azide (Aldrich); 0.0625mol/L solution of (TRIS) tris(hydroxymethyl)aminomethane (Sigma); 2%(m/v) solution of oxalic acid (Merck); 10%(v/v) solution of sulfuric acid (Merck).

Sample collection and preparation

Chrome shavings were collected directly from the tannery industry located 8 Km northwest of Maringa County, Parana State, Brazil. Small pieces of blue chrome shavings were dried at

room temperature, exposing directly to the sun during 5 or 6 h. The preliminary washing step was performed with 2% (m/v) solution of oxalic acid in thermostatic bath at 50°C and 2 h with shaking (Kimura et al, 1996). After removal of chromium, the leather solubilization by chemical and enzymatic methods were performed according to the Figures 1 and 2.

Sun dried chrome shavings (2.500g) were transferred to the 250 mL flask with 75 mL of distilled water in a thermostatic bath at 50°C. After 2 h, the solid was filtered using qualitative filter paper (Figure 1). A solution of 6 M sodium hydroxide was added to the filtrate (branch B) until pH 10, in order to precipitate Cr(OH)₃. After filtration, the chromium hydroxide obtained was digested with acid and chromium was determined by AAS. The solid residue (branch A) was treated with 3% magnesium oxide (w/v) and 10% sodium carbonate at 25°C for 24 h. The sample was then treated with 1% enzyme at 60°C for 2 h. After this treatment, the chrome shavings were almost dissolved producing a viscous yellow solution. The filtrate was separated in three portions for the following experiments: a) protein determination by Biuret's method (Smith,1985); b) acidic digestion and protein determination by Kjeldhal's method (Standard Methods for the Examination of Water and Wastewater, 1992); c) acidic digestion and chromium determination by AAS, as shown in Figure 1.

The same amount of sun dried chrome shavings were transferred to a 250 mL flask with 75 mL of distilled water at 50°C and left for 2 h (Figure 2). After filtration and separation of filtrate (branch B), the residue (branch A) was washed with 40, 50, and 60 mL of 10% sulfuric acid at 25, 40, 50, and 60°C with shaking time of 30, 60, 90, and 120 minutes respectively. The filtration was performed with separation of a blue residue that after acidic digestion was used for chromium determination by AAS. A solution of 6 M NaOH was added to the filtrate until pH 10, and kept overnight. After filtration, the solid

Cr(OH)₃ was kept at 600⁰C for 2 h, and after acidic digestion the chromium oxide was determined by AAS. Proteins were also determined by Biuret and Kjeldhal's methods after acidic digestion of filtered solutions (Figure 2). The filtrate in branch B was treated in the same way as branch B in Figure 1. The final soluble protein shown were used for peptides separations using gel permeation chromatography, and also for amino acids analysis after acidic hydrolysis.

Chromatographic separation

Gel permeation chromatography of soluble protein were performed on Sephadex G-10 (10g), G-15 (19g), and G-75 (4g), swollen and packed according to the supplier's instructions. An aliquot of 1.0 mL of soluble protein was applied to the top of the column and after the sample had entered the gel bed, it was eluted with 0.02% solution of sodium azide. Absorbance at 235 and 280 nm were measured using 1 cm quartz cubets. The void volume (V₀) was determined from the elution of 0.4% solution of Blue Dextran, reading the absorbance at 620 nm.

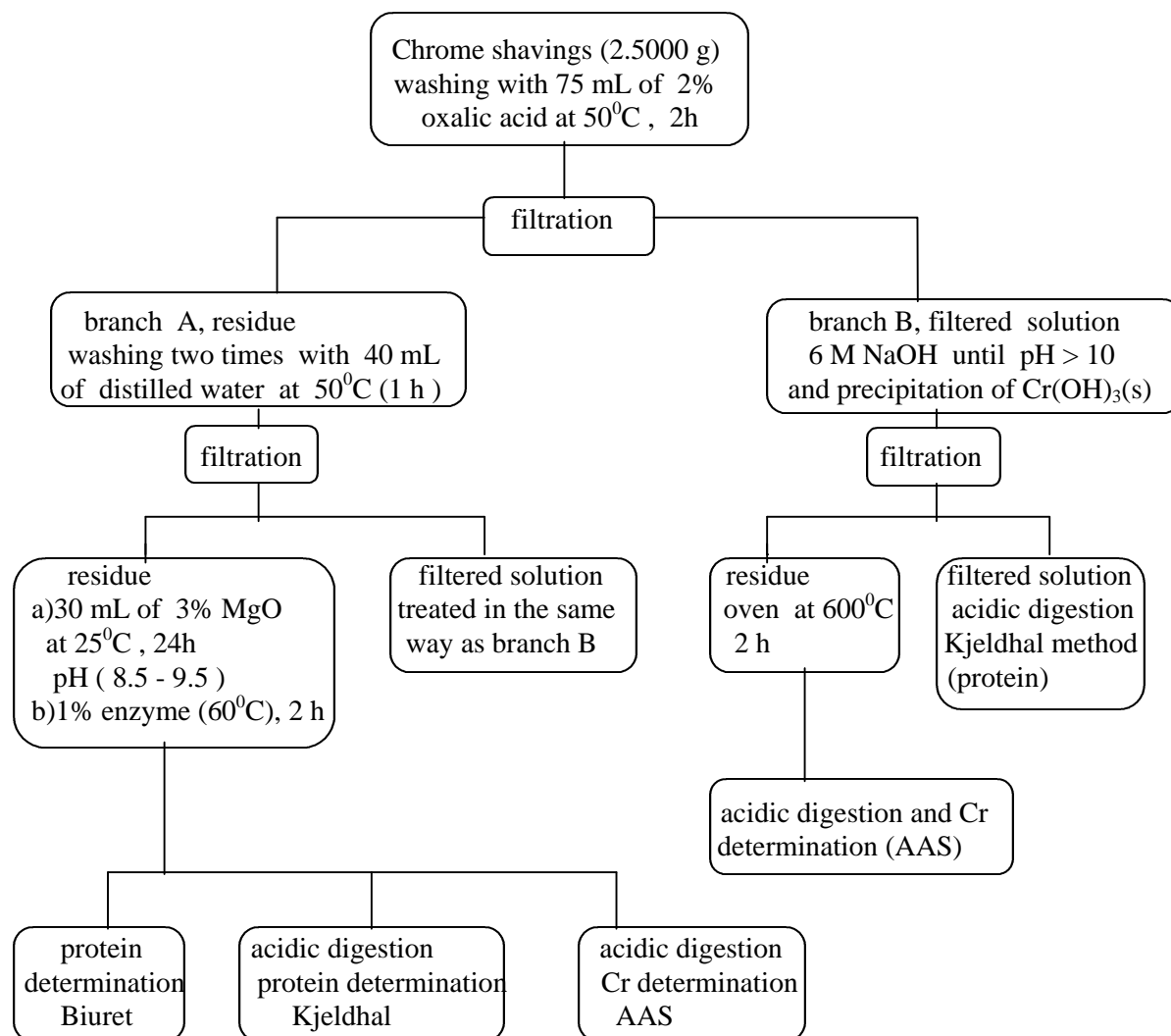


Figure 1. Chrome shavings hydrolysis with proteolytic enzyme and preliminary washing with oxalic acid.

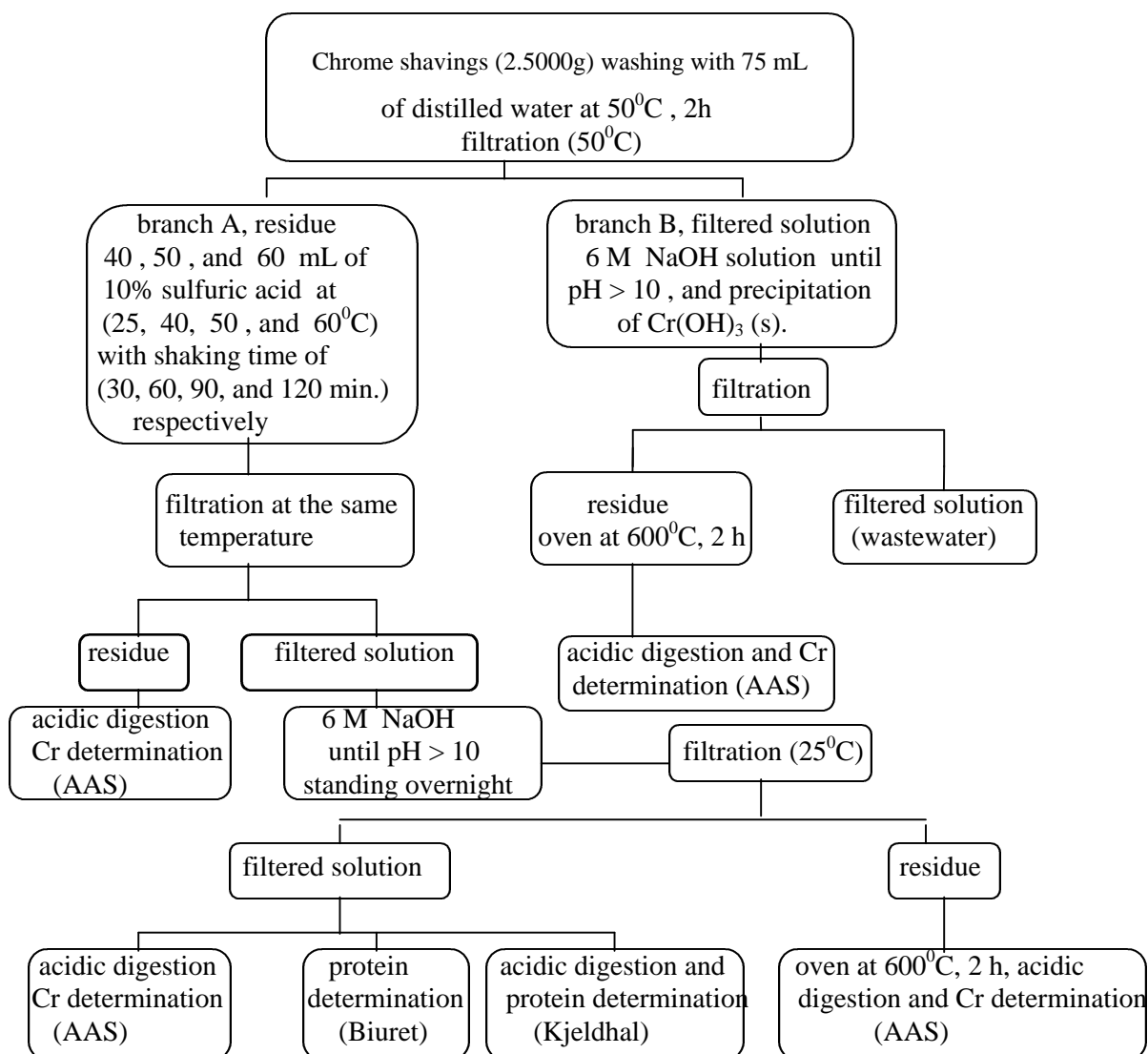


Figure 2. Schematic diagram of chrome shavings hydrolysis using sulfuric acid instead of proteolytic enzyme

The total volume (V_t) was determined with a 30 $\mu\text{L}/\text{mL}$ acetone, reading the absorbance at 264 nm. Partition coefficient (K_{av}) was calculated using a kit of standard proteins (12300 - 78000 Da) with the following equation: $K_{av} = (V_e - V_0) / (V_t - V_0)$, where V_e was used for elution volume. Plot of $K_{av} \times$ logarithm of molecular weight was linear for molecular weight (Daltons) of 16949 (myoglobin), 30000

(carbonic anhydrase), 42700 (ovalbumin), 66250 (albumin), and 78000 (ovotransferrin).

Sample preparation and amino acids analyses by high performance liquid chromatography (HPLC)

The hydrolysis of soluble protein was performed using a Teflon tube with an internal volume of 75 mL by heating at 110°C for 36 h with 6

mol/L HCl solution. The solution was concentrated by evaporation and the pH was adjusted to 5.0 - 5.5. HPLC separations were carried out using sodium cation exchange columns at 55°C (flow rate 0.3 mL/min.). Ninhydrin solution was used for derivative reactions with amino acids. The eluents used were: mobile phase A: buffer solution of 0.2 mol/L sodium citrate, pH 3.25., mobile phase B: buffer solution of 0.01 mol/L sodium phosphate, pH 7.40. Post-column temperature was 130°C with the absorbance reading at 570 nm.

RESULTS AND DISCUSSION

Figures 1 and 2 show the schematic diagram for chrome shavings washing and hydrolysis. Enzymatic (proteolytic enzyme) and acidic (sulfuric acid) were used for complete hydrolysis of chrome shavings (Kimura et al., 1996). Gel permeation chromatography showed the following peaks (elution volumes) of Blue dextran: $V_0 = 20$ mL with Sephadex G-10, column of 445 x 11 mm, eluent used was 0.02% sodium azide solution, flow rate of 0.8 mL/min, $\lambda = 620$ nm. V_0 was also 20 mL for Sephadex G-15 under the same conditions. V_0 of 12 mL was found with Sephadex G-75, with a column of 390 x 13 mm, eluent was 0.02% sodium azide solution, flow rate of 0.1 mL/min., and $\lambda = 620$ nm.

Figure 3 shows chromatographic separation of soluble protein after acidic hydrolysis with sulfuric acid. Figure 4 shows the peptides separations after enzymatic hydrolysis. The volume of 19 mL shown in both figures indicated the presence of peptides of molecular weight higher than 700 Da (Tayyab et al., 1991). The volume (peak) of 35 mL that appeared in Figure 3, but not shown in Figure 4, indicated the presence of peptides with lower molecular weight, and also free amino acids (Whitaker et al., 1963). This was an indication that enzymatic

hydrolysis produced higher amounts of high molecular weight peptides. The same results were observed using Biuret and Kjeldhal's methods for peptides analyses (Kimura et al., 1996).

Figures 5 and 6 show the gel chromatographic separation of soluble protein with Sephadex G-75 after acidic and enzymatic hydrolysis of chrome shavings. Figure 5 shows a peak at 34 mL for peptides of molecular weight higher than 3000 Da, and another peak at 45 mL not shown in Figure 6, for peptides lower than 3000 Da. Figure 6 shows a peak at 17 mL for peptides of molecular weight of 51000 Da (Condell et al., 1993).

Table 1 shows the molecular weight of standard proteins, V_e , and K_{av} calculated as discussed previously. Table 2 shows the amino acids content after solubilization of chrome shavings, and acidic hydrolysis of soluble protein. The contents of amino acids found in this work by HPLC were closely related to the values found by other authors (Table 2) (Reis et al., 1989; Boushy et al., 1991; Taylor et al., 1991). The values found by Tingda (1992), were lower, except for cystine and tyrosine, probably due to the methods employed for chrome shavings solubilization.

CONCLUSIONS

The products recovered after enzymatic hydrolysis of chrome shavings were peptides of higher molecular weight when compared with acidic hydrolysis. Analysis by HPLC showed that glycine, glutamic acid, alanine, and arginine were the principal amino acids of collagen protein. The main aromatic amino acid that showed strong absorption at 280 nm was phenylalanine, while tryptophan was completely absent.

Table 1. Molecular weight, volume of eluent (V_e , mL), and K_{av} of standard proteins.

Protein	molecular weight (Daltons)	V_e (mL)	K_{av}
Cytochrome C	12384	38	1.00
Myoglobin	16949	24	0.46
Carbonic anhydrase	30000	20	0.31
Ovalbumin	42700	18	0.23
Albumin	66250	16	0.15
Ovotransferrin	78000	14	0.08

Experimental conditions: Column of (390 x 13 mm), Sephadex G-75, eluent used was a buffer solution of TRIS-HCl (pH 6.8), and flow rate of 0.1 mL/min. The V_0 used was 12 mL found using Blue dextran. The total volume V_t used was 38 mL measured using acetone.

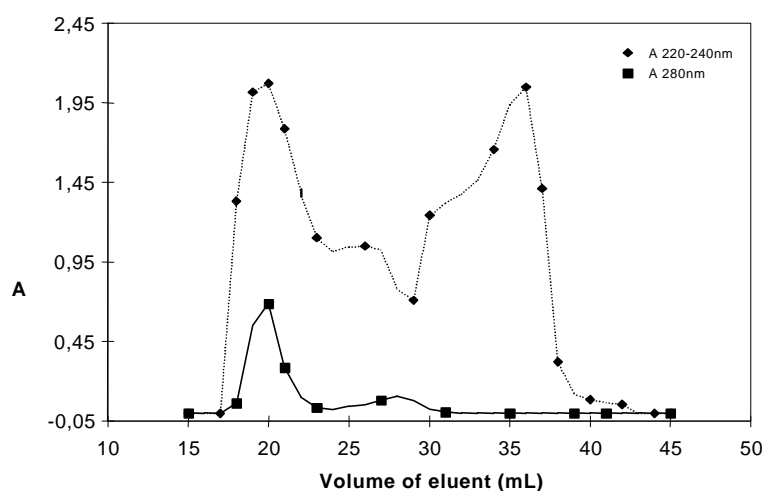


Figure 3: Separation using Sephadex G-10 of soluble protein treated with sulfuric acid. Absorbance x volume of eluent. Dashed line ($\lambda = 235$ nm), black line ($\lambda = 280$ nm).

Experimental conditions: Eluent (0.02 % sodium azide solution). Column: Sephadex G-10 (40-120 μ m),(44.5x1.3cm). Flow rate: 0.8mL/min.

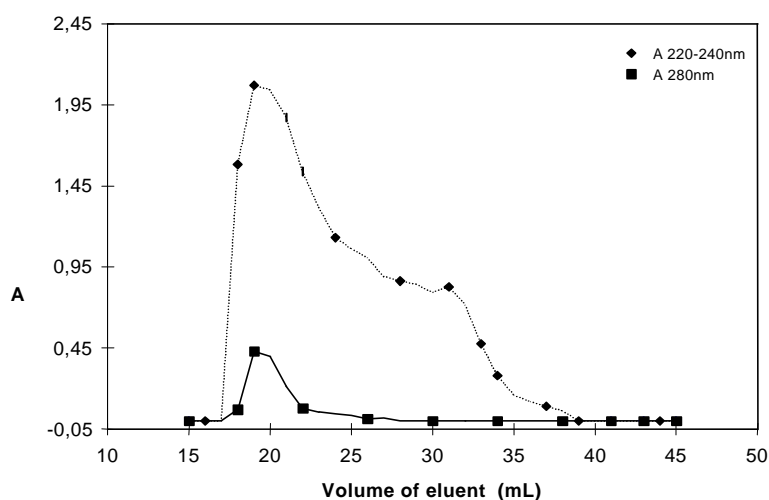


Figure 4: Separation using Sephadex G-10 of soluble protein treated by enzymatic method. Absorbance x volume of eluent. Dashed line ($\lambda = 235$ nm), black line ($\lambda = 280$ nm).

Experimental conditions: Column: Sephadex G-10 (40-120 μ m), (44.5x1.3cm). Eluent: sodium azide solution (0.02%). Flow rate: 0.8mL/min.

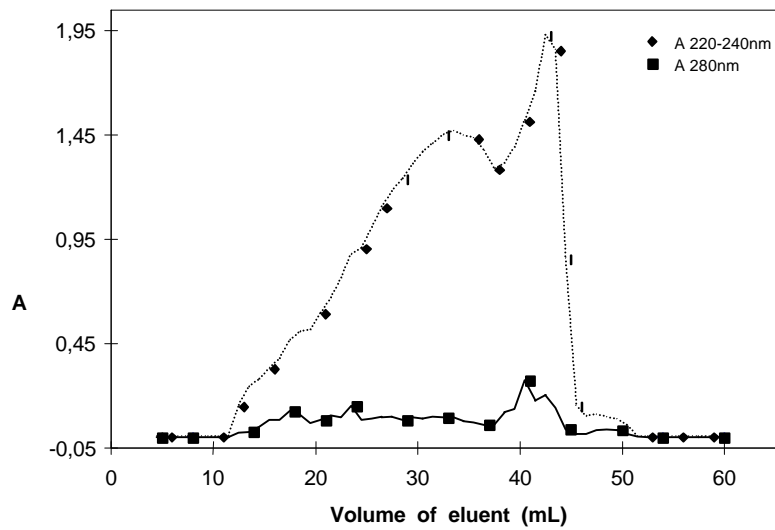


Figure 5: Separation using Sephadex G-75 of soluble protein treated with sulfuric acid. Absorbance x volume of eluent. Dashed line ($\lambda = 235 \text{ nm}$), black line ($\lambda = 280 \text{ nm}$).
 Experimental condition: Column: Sephadex G-75 (20-50 μm), (39.0x1.3cm).
 Eluent: Sodium azide solution (0.02%). Flow rate: 0.1mL/min.

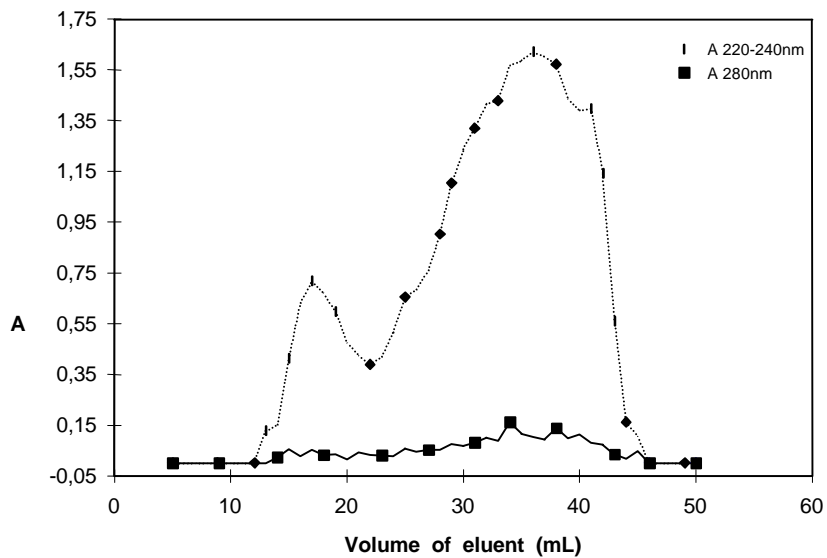


Figure 6: Separation using Sephadex G-75 of soluble protein treated by enzymatic method. Absorbance x volume of eluent. Dashed line ($\lambda = 235 \text{ nm}$), black line ($\lambda = 280 \text{ nm}$).
 Experimental condition: Column: Sephadex G-75 (20-50 μm), (39.0x1.3cm).
 Eluent: Sodium azide solution (0.02%). Flow rate: 0.1mL/min.

Table 2. Amino acids contents in chrome shavings in % related to the dry leather.

Amino acids	A	B	C	D	E
Aspartic acid	4.6	2.9	4.7	5.1	4.3
Threonine	1.8	0.8	0.8	2.1	1.3
Serine	3.2	1.4	1.3	4.1	2.6
Glutamic acid	10.6	5.6	9.0	7.7	7.3
Phenylalanine	1.8	1.1	1.8	1.3	1.4
Histidine	0.7	0.4	0.5	0.9	0.6
Glycine	17.0	10.6	20.5	33.0	14.8
Alanine	9.2	4.5	9.4	8.4	6.4
Cystine	0.1	0.3	ND	ND	ND
Valine	2.2	1.5	2.1	2.4	1.7
Lysine	3.7	1.5	4.1	2.7	2.6
Arginine	8.2	3.4	6.5	4.8	5.9
Methionine	1.3	0.3	1.0	0.2	0.6
Isoleucine	1.4	0.9	1.3	1.4	1.2
Leucine	2.8	1.6	2.8	2.6	2.2
Tryptophan	ND	0.1	ND	ND	ND
Tyrosine	0.3	0.6	0.4	0.5	0.5

A) Represents the amino acids determined in this work by HPLC.

B) Amino acids found by Tingda et al.

C) Represents the work of Reis et al.

D) Taylor et al.

E) Boushy et al.

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RESUMO

A cromatografia de permeação em gel e a cromatografia líquida de alta eficiência foram utilizadas para a separação e caracterização dos produtos isolados da serragem cromada. Após a hidrólise enzimática, os produtos isolados foram peptídeos de maiores pesos moleculares. Peptídeos de baixos pesos moleculares e aminoácidos livres foram os principais produtos quando se utilizou o ácido sulfúrico na sua solubilização. Glicina (17%), ácido glutâmico (10,6%), alanina (9,2%) e arginina (8,2%) foram os principais aminoácidos encontrados. O principal aminoácido aromático foi a

fenilalanina (1,8%) com a ausência completa do triptofano.

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