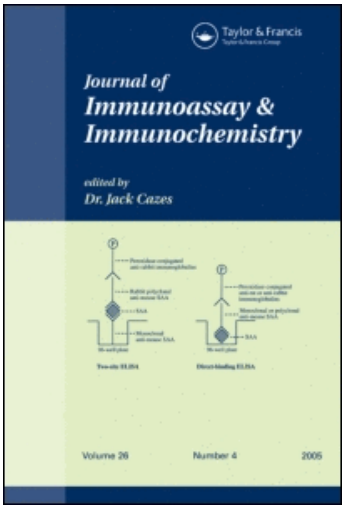

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A NEW METHOD FOR BOVINE PEPSINOGEN PURIFICATION. PREPARATION OF A SPECIFIC ANTIBODY.

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ABSTRACT

Bovine pepsinogen was purified from abomasum by ammonium sulphate precipitation and ionic exchange chromatography on DEAE-cellulose and Mono Q columns. Purified pepsinogen was shown to be homogeneous by analytical electrophoresis, having an estimated molecular mass of 46,000 Daltons. The isoeletric point, determined by analytical chromatofocusing was 4.6. Using this pepsinogen preparation to immunize rabbits, a specific antiserum of high titer was obtained.

KEY WORDS - Bovine pepsinogen, pepsinogen antiserum, pepsinogen ELISA.

INTRODUCTION

Gastrointestinal parasites are among several factors that have contributed to problems encountered in Brazilian cattle husbandry. They result in major

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economic losses due to morbidity and mortality. Some parasites, during their histotrophic phase may damage the abomasa wall leading to a greater permeability to macromolecules like pepsinogen that may reach the blood stream (1). Therefore, the blood pepsinogen level may reflect the morphological and functional status of the gastric mucosa and will be a better criterion than fecal egg counts or antibody titer for the diagnosis of bovine ostertagiasis. Elevated serum pepsinogen levels reflect larval availability, abomasal damage and the development of large adult worm burdens (2-6). However, majority of the data on bovine pepsinogen levels has been reported in terms of the proteolytic activity of the acid-activated pepsin present in the sample. Pepsinogen levels in serum can be determined more reliably by radioimmunoassay or enzyme linked immunosorbent assay (ELISA). For using both these techniques, the major problem is the availability of anti-pepsinogen antiserum, the preparation of which requires large quantities of highly purified pepsinogen. Bovine pepsinogen has been purified by different methods involving several steps of conventional chromatography (7,8). However, the yield is generally low due to the high instability of pepsinogen which is slowly activated to pepsin below pH 6.0 and denaturated above pH 7.8.

In this paper, we are presenting results of a simple and high recovery procedure for bovine pepsinogen purification. A procedure for preparation of high titer anti-pepsinogen antiserum is also described.

MATERIALS and METHODS

Materials

DEAE-cellulose was from Whatman. Mono Q HR 5/5, Mono P HR 5/20 and polybuffer 74 were from Pharmacia. Goat anti-rabbit immunoglobulins

peroxidase conjugate was from Bio Rad and porcine pepsin was from Sigma. All other chemical products were of analytical grade.

Purification procedure

All steps of the purification procedure were carried out at 4° C. Fresh bovine abomasa, obtained from slaughter-house were dissected and the mucosa homogenized in 0.1 M sodium phosphate buffer pH 7.3 (3 mL/g) and the homogenate centrifuged for 60 minutes at 15,000xg. The supernatant was brought to 42% saturation with solid ammonium sulphate and after stirring for 30 minutes, the precipitated material was removed by centrifugation at 10,000xg for 30 minutes and discarded. Solid ammonium sulphate was added to the supernatant to achieve 70% saturation and after stirring for 30 minutes the precipitate was collected by centrifugation, dissolved in a minimal volume of 20 mM sodium phosphate buffer pH 7.0, dialysed for 12 h against phosphate buffer (2 L) and applied onto a DEAE-cellulose column (2.0 x 9.0 cm). The proteins were eluted firstly washing isocraticaly the column with phosphate buffer containing sodium chloride at following concentrations: 20, 50, 75, 100 and 200 mM, secondly by a sodium chloride linear gradient (200-500 mM) and finally isocratically at 500 mM of the sodium chloride in the phosphate buffer. The fractions containing pepsinogen were pooled, dialysed for 12 h against 20 mM sodium phosphate buffer pH 7.0 (2 L) and injected onto a Mono Q column equilibrated with the phosphate buffer. The chromatography was carried out at a flow rate of 1.0 mL/min by a combination of the following elution modes: step 1, isocratic elution with 20 mM sodium phosphate buffer pH 7.0 for 5 minutes and step 2, a linear sodium chloride gradient (0 - 1.0 M) in the phosphate buffer for 25 minutes.

Pepsinogen assay

Pepsinogen was determined by assay of the proteolytic activity after acid treatment utilizing hemoglobin as substrate as previously described by Kassell and Meitner, 1970 (8). Briefly, for 0.9 mL of 2.5% (w/v) hemoglobin dissolved in 0.01 M HCl, 0.1 mL of the sample to be assayed is added and the incubation carried out for 10 minutes at 37° C. The reaction was terminated by addition of 2 mL of 5% (w/v) trichloroacetic acid. After centrifugation, the absorbance of the supernatant was determined at 280 nm. The activity unit (AU) is defined as the increase of 0.001 units per minute in the absorbance.

Pepsin assay

To detect the presence of pepsin in the purified pepsinogen preparation, the milk-clotting assay was used. The procedure was as described by Kassell and Meitner, 1970 (8).

Immunization procedure

Anti-pepsinogen antisera were raised in New Zealand white rabbits by injection of 2 mg of purified pepsinogen in complete Freund's adjuvant at multiple subcutaneous sites in the back. Booster of 2 mg of pepsinogen in complete-incomplete Freund's adjuvant (1:1) was given one month later at multiple subcutaneous sites in the back. For the subsequent boosters, 1 mg of pepsinogen was emulsified in incomplete Freund's adjuvant and injected as described above. Blood was collected monthly, 24 h after booster, from the marginal ear vein. The titers of the various collected sera and the cross-reactivity with pepsin were determined by ELISA and by quantitative precipitin test (9).

Enzyme-linked immunosorbent assay

The assay was carried out in polystyrene microtritation plates. For the determination of antisera titers, microtiter wells were coated with antigen (0.5 ug of purified pepsinogen or pepsin per well) in 100 uL of 100 mM sodium carbonate buffer pH 9.6 and kept overnigth at 4° C. The wells were then washed three times with 0.1 M sodium phosphate buffer pH 7.2, containing 0.15 M NaCl (PBS) plus 0.05% (v/v) Tween 20 (PBST). This was followed by blocking of nonspecific binding with 100 uL per well of 0.3% (w/v) casein in PBS (PBSC) for 60 minutes at room temperature. The wells were washed again with PBST, and incubated with serial dilutions of rabbit antibodies. All immunoglobulins were diluted in the range 1:40 to 1:5,120 with PBS containing 0.3% (w/v) casein. The plates were washed as above and 100 uL of peroxidase conjugate goat anti-rabbit IgG diluted in PBSC 1:3000 were added to each well and incubated as above for 60 minutes. After five washings with PBST, 100 uL of 0.002% (w/v) O-phenylenediamine in citrate-phosphate buffer pH 5.0 (46 mM Na_2HPO_4, 100 mM citric acid) containing 0.012% (v/v) H_2O_2 was added to each well. The reaction was interrupted after 30 minutes at room temperature by the addition of 30 uL of 2 M H_2SO_4. The absorbance was measured at 492 nm wavelength. The titer was defined as the antiserum dilution corresponding to the mean optical density plus two standard deviations of the negative control. For negative control, rabbit normal serum was used. For the determination of the sensitivity of the antisera, the microtiter wells were coated with pepsinogen so that each well contained the antigen in the range 1,000 to 32 ng and incubated with 100 uL of antisera diluted 1:2,000. All procedures were as described above.

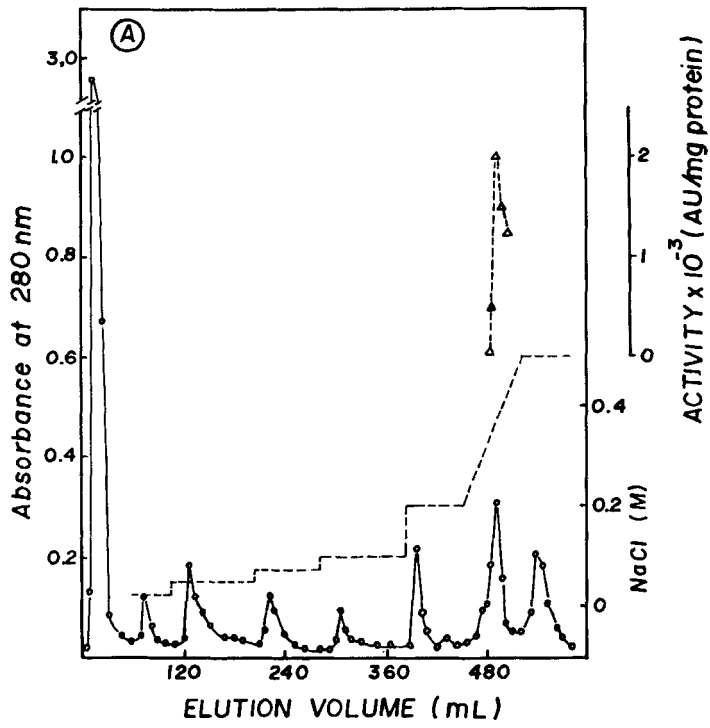

Figure 1 - Elution profile obtained from ionic exchange chromatography on: A) DEAE-cellulose and B) Mono Q. The columns were equilibrated with 20 mM sodium phosphate buffer pH 7.0 and the proteins eluted with a NaCl gradient shown by the dashed lines. The pepsinogen activity (Δ) was determined utilizing hemoglobin as substrate as described in materials and methods.

Analytical chromatofocusing on Mono P column

Pepsinogen obtained from the Mono Q chromatography was applied on a Mono P HR 5/20 column for pI determination. Previously, the column was equilibrated with 25 mM bis-Tris-HCl buffer pH 6.5. After loading with the sample, the column was eluted with a 1:10 diluted solution of the polybuffer 74, pH 4.0 for 40 minutes in a flow rate of 1 mL/min.

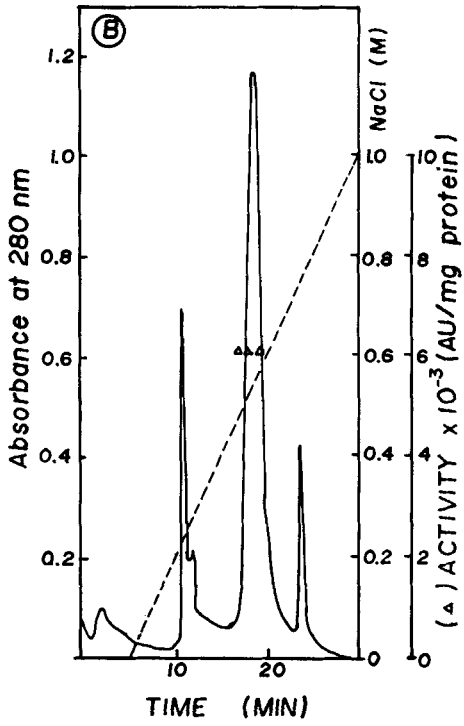

Figure 1 Continued

Electrophoresis

Sodium Dodecyl Sulphate (SDS)-Polyacrylamide Gel Electrophoresis, was performed as described by Laemmli, 1970 (10), in 10% acrylamide gels with 0.1% (w/v) SDS cast in 0.375 M Tris-HCl buffer pH 8.8 as a separating gel and 4% gel cast in 0.125 M Tris-HCl buffer pH 6.8 with 0.1% (w/v) SDS as stacking gel. Purified pepsinogen (1 - 5 ug) was treated with 20 uL of 0.15 M Tris-HCl buffer pH 6.8 containing 2.9% (w/v) SDS, 5.8% (v/v) glycerol and 2.9% (v/v) 2-mercaptoethanol and boiled for 3 minutes. Electrophoresis was carried out at room

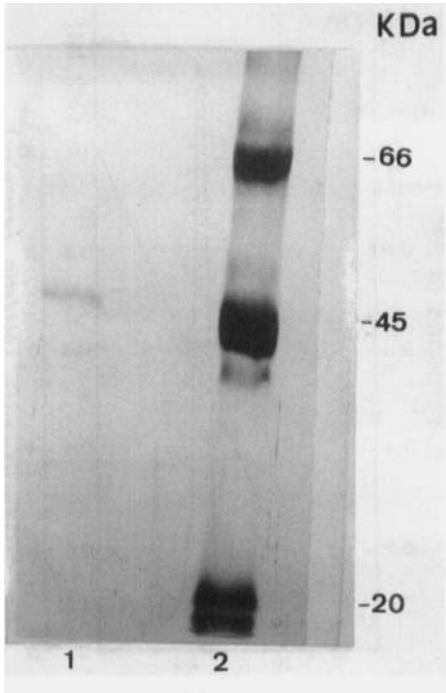

Figure 2 - SDS-Polyacrylamide gel electrophoresis of the pepsinogen (lane 1) and molecular mass markers (lane 2).

temperature with a constant voltage of 100 V, using 20 mM Tris-glycine buffer pH 9.0 containing 0.1% (w/v) SDS. Afterward, the gel was fixed first with an aqueous solution containing 43% (v/v) methanol and 7% (v/v) acetic acid for 1 h and then with an aqueous solution containing 10% (v/v) methanol and 10% (v/v) acetic acid for 1 h. For staining the gel was soaked for 30 minutes in a 0.09 M NaOH solution containing 1.4% (v/v) NH_4OH and 0.4% (w/v) silver nitrate. The molecular mass markers used were bovine serum albumin (Mr 67,000), ovalbumin (Mr 45,000), rat tonin (Mr 29,000) and soybean trypsin inhibitor (Mr 20,100).

TABLE 1

Purification of abomasa bovine pepsinogen from 1 g of mucosa.

PROCEDURE	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (AU)	SPECIFIC ACTIVITY*	PURIFICATION	RECOVERY (%)
Homogenate *supernatant*	25.00	2,000	80	1	100.0
DEAE	0.50	897	1,793	22	44.9
Mono Q	0.27	732	6,117	76	36.6

* Activity units (AU)/ milligram of protein.

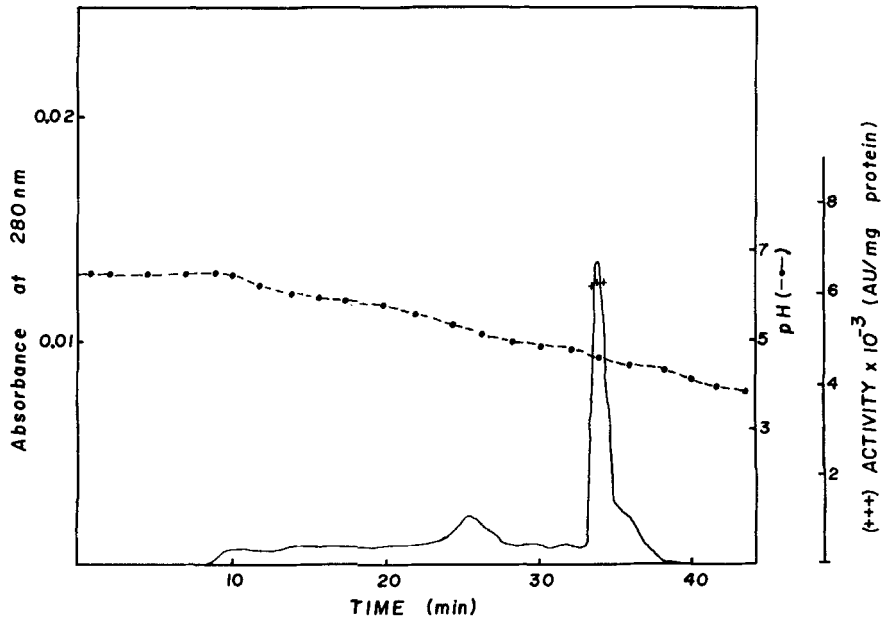

Figure 3 - Elution profile obtained in a Mono P column of the pepsinogen after Mono Q chromatography.

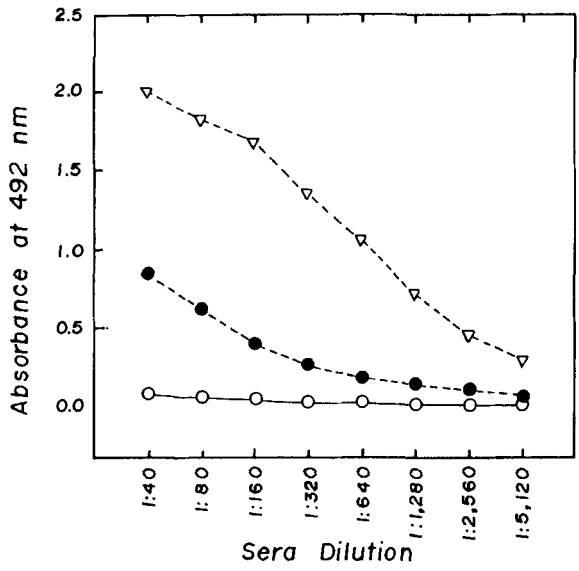

Figure 4 - Comparison of the average reactivity of pepsinogen (Δ) and pepsin (●) with anti-pepsinogen antiserum, determined by ELISA. Plates were coated with 0.5 ug of antigen and incubated with antiserum in a final dilution as indicated. For negative control (O) normal rabbit serum was used.

RESULTS

Pepsinogen

The results of the chromatographic steps of the pepsinogen purification procedure are presented in the figure 1. As can be seen in this figure, only one fraction presenting pepsinogen activity was observed after DEAE and Mono Q chromatography. This pepsinogen preparation appears to be homogeneous by SDS-polyacrylamide gel electrophoresis and has a molecular mass of 46,000 Da (figure 2). At this stage, the pepsinogen is practically free of pepsin. However, after six months of storage at -20° C, approximately 30% of the total activity was

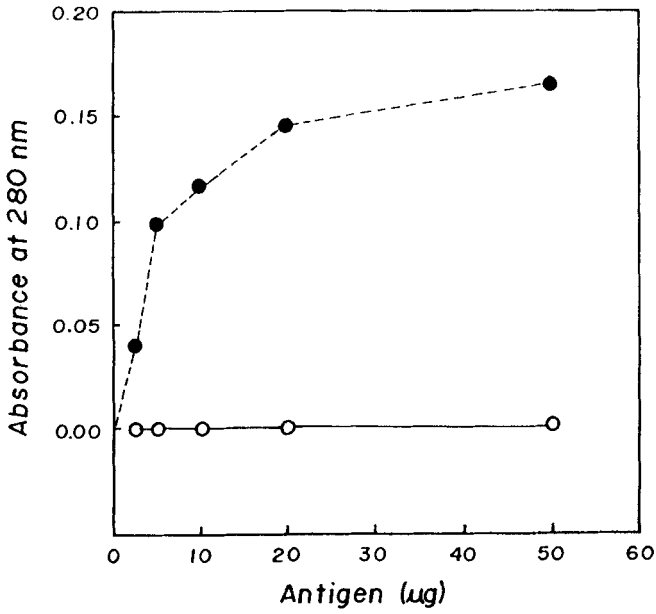

Figure 5 - Precipitin reactions of anti-pepsinogen antiserum with pepsinogen (●) and pepsin (O).

due to pepsin. The specific activity of the purified pepsinogen was 6,117 AU per milligram of protein. Other parameters related to the purification procedure are in the table 1.

The isoeletric point of the bovine pepsinogen, obtained by analytical chromatofocusing using a Mono P column is shown in the figure 3. The elution profile showed in this figure presents one peak of protein which coincided with that of pepsinogen activity and eluted at pH 4.6.

Anti-pepsinogen antisera

Three months after first injection, sera collected from rabbits had titers of 1:5,120 and 1:160 for pepsinogen and pepsin, respectively as determined by

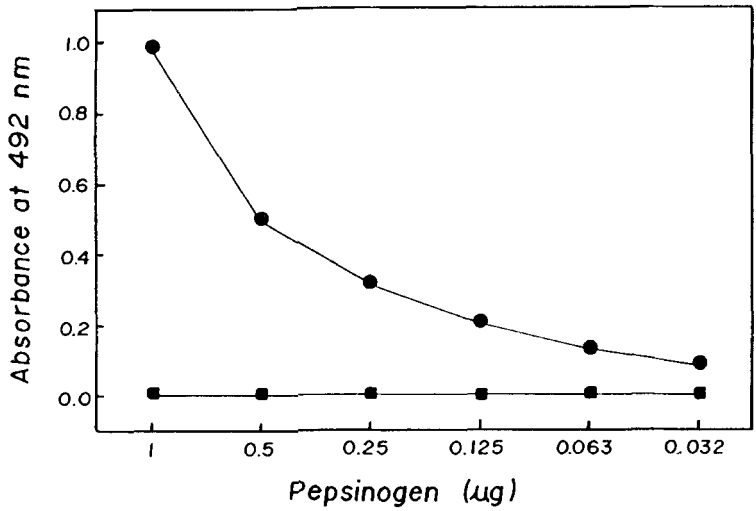

Figure 6 - Reactivity of pepsinogen (●) with anti-pepsinogen antiserum (1:2,000) in ELISA. For negative control (■) normal rabbit serum (1:2,000) was used.

ELISA. These results, presented in figure 4, show the high specificity of these antisera since its cross-reactivity with pepsin is very low. The low cross-reactivity with pepsin was also observed by quantitative precipitin test (figure 5). The sensitivity of these antisera was determined by ELISA. At 1:2,000 dilution as little as 32 ng of pepsinogen may be detected (figure 6).

DISCUSSION

The plasma pepsinogen level may provide an important information for the diagnosis of bovine helmintosis involving destruction of the abomasa wall. Generally it has been used for diagnosis of ostertagiasis. Little is known about the bovine plasma pepsinogen levels in animals presenting with other gastrointestinal

helmintosis. This may be explained by the difficulty observed in the pepsinogen purification, since pepsinogen is very susceptible to denaturation and activation to pepsin. Bovine pepsinogen was first purified from mucosa of abomasa in 1968 by Chow and Kassell (7), by a procedure involving five chromatographic steps with a yield less than 15%. Approximately 95 mg of pepsinogen were obtained from 9.1 kg of mucosa. The procedure presented in this study is an improvement of the pepsinogen purification since it is obtained in a homogeneous form by two chromatographic steps and therefore the recovery was relatively higher. Taking into account the recovery of 36.6% and that the total protein of the purified preparation is due to pepsinogen, we may conclude that 1 g of bovine mucosa contains approximately 0.74 mg of pepsinogen. The molecular mass observed by Chow and Kassell, 1968 (7) for the bovine pepsinogen was 38,900, determined by amino acid analysis and 37,500, determined by ultracentrifugation. The molecular mass obtained by us using polyacrylamide gel electrophoresis was 46,000. Probably, there are different forms of bovine pepsinogen. The purified pepsinogen is very immunogenic since after three months injection in rabbits, large amounts of an antibody with high titer was obtained. Similar results were obtained for porcine pepsinogen by Bustin and Conway-Jacobs, 1971 (9). This anti-pepsinogen antiserum is highly specific and shows low cross-reactivity with pepsin. This antiserum can be used for a sensitive assay of pepsinogen, since at 1:2,000 dilution in ELISA, as little as 32 ng of pepsinogen may be detected. As specificity and sensitivity are important factors that determine the characteristics of an antibody to be used in a quantitative assay, this antiserum may be used for the diagnosis of helmintosis which may alter the plasma pepsinogen levels.

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