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Pepsinogen Activity

Determination in Serum and Plasma

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1. Introduction

Pepsin is a proteolytic enzyme (EC 3.4.4.1) present in the gastric secretions of mammals. Pepsin is derived from an inactive precursor, pepsinogen, produced principally in the Chief cells of the gastric mucosa. Under acid conditions, cleavage of a peptide from the N-terminal end of pepsinogen occurs by an autocatalytic process to form the active pepsin enzyme (Schillhorn, 1988).

Certain forms of gastric mucosal damage are associated with increased blood concentrations of pepsinogen. In ruminants, serum or plasma concentrations of pepsinogen provide a useful indicator of abomasal damage associated with the larval forms of *Ostertagia* infection. Pepsinogen estimation is particularly indicated in adult cattle, where faecal egg counts may be poorly correlated to nematode burden (Kloosterman *et al.*, 1984).

Pepsinogen should be used as a herd rather than individual animal test, as individual animals may give false negative results (Schillhorn, 1988). Low blood pepsinogen concentrations have also been observed in animals with chronic abomasal damage.

2. Principle of the Pepsinogen Assay

Serum is added to a bovine serum albumin (BSA) substrate solution containing glycine ($C_2H_5NO_2$) buffer at pH 1.6. During incubation at 37°C, pepsin hydrolyses the substrate, releasing peptide fragments. After three hours., residual substrate is precipitated with perchloric acid ($HClO_4$) and the peptide fragments remaining in the supernatant are measured using a modified Folin reagent. Activities are calculated by comparison with a series of tyrosine ($C_9H_{11}NO_3$) standards, and subtraction of appropriate serum blanks.

3. Materials Required for the Pepsinogen Assay

3.1. Samples

Serum, heparinised or ethylenediamine-tetraacetic acid (EDTA) plasma. Haemolysis has a negligible effect on activity determined by this method. Activity in serum/plasma is stable for at least several days at 4°C, and for at least several months at -20°C.

3.2. Chemicals

(a) BSA (Dried), from Commonwealth Serum Laboratories (CSL, Parkville, Vic. 3052, Australia.
(b) Glycine, analytical reagent (AR) grade.
(c) Hydrochloric acid (HCl), 31.5% w/w, AR grade.
(d) L-tyrosine, chromatographically homogenous, e.g. BDH, Calbiochem.
(e) Perchloric acid, 70% AR grade.
(f) Pierce BCA protein reagent, Pierce Cat. No. 23225 [Pierce Chemicals, PO Box 117, Rockford, Ill 61106, USA. Tel. (800) 874 3723].

3.3. Reagents

3.3.1. Pepsinogen Substrate

The pepsinogen substrate is 3.2% BSA (w/v) in glycine (0.2 mol/L) at pH 1.6.

Add 3.2 g BSA and 1.0 g glycine (with stirring) to 90 mL of distilled water. Titrate with hydrochloric acid (31.5%) to pH 1.6. Adjust final volume to 100 mL, and readjust pH to 1.6 if necessary.

Store in 5 mL aliquots at -20°C. Stable for several months at -20°C.

3.3.2. Perchloric Acid 2% (v/v)

Add 20 mL of perchloric acid (70%) to 980 mL of water.

3.3.3. Perchloric Acid 10% (v/v)

Add 100 mL of perchloric acid (70%) to 900 mL of water. Stable for several months at room temperature.

3.3.4. Tyrosine Standards

3.3.4.1. Primary standard (10 mmol/Ltyrosine)

Add 181 mg of L-tyrosine to 2% perchloric acid, to a final volume of 100 mL.

3.3.4.2. Working standards

Prepare the following dilutions in 10 mL plastic tubes.

(a) 0 mL Primary standard + 10.0 mL 2% (v/v) perchloric acid (0 mmol/L tyrosine = 0 U/L).
(b) 0.9 mL Primary standard + 9.1 mL 2% (v/v) perchloric acid (0.9 mmol/L tyrosine = 5 U/L).
(c) 1.8 mL Primary standard + 8.2 mL 2% (v/v) perchloric acid (1.8 mmol/L tyrosine = 10 U/L).
(d) 3.6 mL Primary standard + 6.4 mL 2% (v/v) perchloric acid (3.6 mmol/L tyrosine = 20 U/L)
(e) 5.4 mL Primary standard + 4.6 mL 2% (v/v) perchloric acid (5.4 mmol/L tyrosine = 30 U/L).

Working standards are stable for several months at 4°C.

3.3.5. Protein Reagent

Pierce BCA Protein Reagent. Prepare the working protein reagent from the two separate reagents, as described in the package insert.

4. Procedure for the Assay of Pepsinogen Activity

4.1. Incubation

To 1 mL Eppendorf tubes, add: 0.075 mL of pepsinogen substrate; and 0.050 mL of serum, serum controls or tyrosine standards.

For serum and serum controls, two tubes are required per sample, labelled N and I.

Cap all tubes, mix on Vortex mixer. Incubate all tubes at 37°C. After 30 min, add 0.25 mL of perchloric acid (10%) to all N tubes and to tubes with tyrosine standards. Incubate all tubes for further three hours (i.e. three hours 30 min total incubation time), then add 0.25 mL of perchloric acid (10%) to I tubes. Mix. Cool to 4°C.

Centrifuge all tubes at 2000 *g* (minimum) for 10 min.

4.2. Colour Development

In 7 mL test tubes, add 0.05 mL of perchloric acid supernatant to 1.0 mL of Pierce BCA Protein Reagent. Incubate at 37°C for 30 min. Measure absorbance of the solution at 562 nm. The colour development part of this assay can be automated. Where the chemical autoanalyser is equipped with sample liquid sensing facilities, the three hour pepsinogen incubation and subsequent protein precipitation step, may be conveniently performed directly in the appropriate autoanalyser sample cup, eliminating the requirement for decanting supernatents from each assay.

5. Calculations

Calculate activities for both N and I incubations of each sample, by direct comparison with the standard curve.

U/L = µmol of tyrosine released/min per L serum
= [(U/L in I) – (U/L in N)]

6. Interpretation of Results

The following values have been established for bovines at Benalla Regional Veterinary Laboratory (Table 1). *N.B.* False negatives may occur. Pepsinogen values should be interpreted on a herd rather than on an individual basis.

7. Notes on the Assay

(a) Quality controls suitable for this assay are not commercially available. To monitor between and within-run assay variation, it is recommended that bulked serum samples (one of high activity and one of low activity) are prepared, aliquoted and stored at -20°C. Activities of these samples should be checked by interlaboratory comparison.

(b) Non-enzymatic rate of reaction is negligible for the period of incubation used, and can be omitted from activity calculations.

(c) Reaction rates are linear over at least a six-hour incubation period, for activities within the range of standards, after an initial 30 min lag period. A proportion of this 30 min lag period appears to be due to the autocatalytic conversion of pepsinogen to its active pepsin form.

(d) Detection limit of the assay is approximately 0.2 U/L. This corresponds to an absorbance of approximately 0.003 at 562 nm.

(e) Colour development using the Pierce BCA reagent is time-dependent, and proceeds at a slow rate after the 30 min incubation period at 37°C. To minimise this effect, absorbances should be read as quickly as possible, or the colour development period between addition and final readings timed. For routine work, the former is found to be satisfactory.

(f) The assay pH used (pH 1.6 for substrate, giving a final assay pH of 2.4), is optimal for bovine and ovine pepsin activity. A single pH optimum is observed for serum pepsin activity in both these species.

(g) High blank (N tube) values are observed with serum from some animals. These high blanks appear to be due to acute phase protein elevations, with some of these proteins not being precipitated by perchloric acid at the concentrations used in this assay (Nagahata *et al.*, 1989). Incubation of N tubes at 37°C, after the addition of perchloric, during the period that the I tubes are also being incubated, minimises interference of these proteins in the pepsinogen assay.

Table 1. Interpretation of pepsinogen values in bovine serum

U/L	
<5	No significant abomasal damage
5–10	Minor abomasal damage
10–15	Moderate abomasal damage
>15	Major abomasal damage

8. References

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