

## Equine trypsin: purification and development of a radio-immunoassay

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**Abstract** – Shock is accompanied by generalised splanchnic hypoperfusion, and splanchnic organs like the pancreas can be damaged, as shown in animal experimental models and in humans, by the presence of high plasma concentrations of trypsin and other pancreatic enzymes. In order to design a radioimmunoassay technique (RIA) for the measurement of equine trypsin-like immunoreactivity (TLI) in biological fluids, trypsin was purified (with purity  $\geq 96\%$ ) from the equine pancreas by extraction in an acid medium, ammonium sulfate precipitations, gel filtration chromatography and, after activation of trypsinogen into trypsin, affinity chromatography. Gel polyacrylamide electrophoresis showed a monomeric enzyme with a molecular weight of 27 kDa. The purified equine trypsin served for the immunisation of rabbits in order to obtain a specific antiserum, and the labelled antigen was prepared by iodination of equine trypsin with  $^{125}\text{I}$ . The RIA was based on the binding of the antigen to the antibody followed by the separation of the antigen-antibody complex by immunoprecipitation in the presence of sheep anti-rabbit gammaglobulins and the assay of the radioactivity in the precipitate. The RIA showed good sensitivity, specificity, precision, accuracy and reproducibility. The reference mean value of TLI in the plasma of healthy horses ( $n = 20$ ) was  $30.01 \pm 6.84$  ng/mL (upper confidence limit 50.52 ng/mL;  $p < 0.01$ ). Three horses with non strangulating intestinal obstruction without shock showed TLI values within normal limits whereas 5 of 7 horses with strangulation obstruction showed TLI levels above the upper confidence limit. Further studies using the RIA and the enzymatic assay should be performed in order to confirm the role of the pancreas in equine intestinal obstruction.

**equine trypsin / purification / radio-immunoassay / reference range / intestinal obstruction**

### 1. INTRODUCTION

Hypovolaemic and endotoxic shock following intestinal obstruction or severe sep-

sis may lead to generalised hypoperfusion to which several organs (the central nervous system, lungs, heart, kidneys) are very susceptible [28, 31, 39]. Hypoperfusion

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induces a systemic reaction with leukocyte activation and a generalised inflammation that finally leads to multiple organ failure [3]. The organs of the splanchnic area are also very susceptible to hypoperfusion, although often clinically silent. Several experimental studies have shown the particular susceptibility of the pancreas to hypoperfusion with a decrease of capillary blood flow, an increase of pancreatic proenzymes and enzyme-complexes in the general circulation and histo-pathological alterations of the pancreas [12–15, 17, 23]. Similar findings have been made for humans with severe trauma and hypovolaemic or septic shock [6, 22, 35, 47, 50]. Splanchnic hypoperfusion and systemic inflammatory reaction contribute to pancreatic damage, with leukocyte accumulation in the pancreatic capillaries and anoxia of highly active metabolic rate cells of the exocrine pancreas. Recently, digestive enzymes were found to induce the formation of small molecular weight cell activators that may play an important role in the development of microcirculatory dysfunction and multiple organ failure [32].

In horses, strangulating intestinal obstruction often leads to the development of endotoxic shock [34, 41]. Due to the alteration of the intestinal permeability, endotoxins enter the general circulation [33], but the entry of digestive enzymes, recognised as normal (in humans) to some extent [8, 26] can also be increased. The implication of the pancreas or pancreatic enzymes in acute abdominal disease in the horse has not, to date, been well documented. In a previous study, we have shown the presence of high plasma concentrations of active trypsin (probably bound to  $\alpha_2$ -macroglobulin) in horses suffering from strangulating intestinal obstruction and endotoxic shock [19].

However, on the basis of current knowledge concerning complex forms of human and bovine trypsin, we can conclude that the measurement of active trypsin does not account for all the forms of trypsin that

may be present in the general circulation of horses. In biological fluids, trypsin exists under several forms, as an inactive precursor (trypsinogen) and complex forms with  $\alpha_2$ -macroglobulin ( $\alpha_2$ -M) and  $\alpha_1$ -proteinase inhibitor ( $\alpha_1$ -PI) [9, 29, 36, 45]. In physiological conditions, the presence of free trypsin seems nearly impossible because of large amounts of plasmatic inhibitors [37]. The enzymatic assay of trypsin only measures free trypsin and trypsin bound to  $\alpha_2$ -M because this binding does not inhibit the activity of the enzyme but hinders its access for large molecular weight substrates [38, 42]. The specificity of trypsin RIA depends on the antigenic properties of the enzyme but not on its enzymatic activity [45]. The immunological assay, based on the recognition by specific antibodies, thus permits the measurement of trypsin-like immunoreactivity (TLI). TLI is composed of trypsinogen, free trypsin, trypsin bound to  $\alpha_1$ -PI, bound to inter- $\alpha_1$ -trypsin inhibitor and even to low molecular weight inhibitors such as aprotinin or pancreatic secretory inhibitor [15, 16, 24, 45]. Trypsin bound to  $\alpha_2$ -M is buried in a pocket of this large molecule, and is thus not recognised by the antibodies because of steric inhibition [1, 5]. The immunological method is thus complementary to the enzymatic assay and the application of the two methods may determine the entire quantities of trypsin in biological fluids. An immunological assay of equine trypsin has not yet been described, and we only found one abstract concerning its purification [21].

The aim of this study was to describe the purification of equine trypsin and the development of a radio-immunoassay technique (RIA) for equine TLI. The purification was based on the method applied for human trypsin [4, 30, 46] and the RIA on the methods previously published relating to human trypsin [16, 30, 45]. The RIA was applied to equine plasma for the determination of plasma values in healthy horses in order to establish a reference

range. Preliminary results in acute intestinal obstruction are also mentioned.

## 2. MATERIALS AND METHODS

### 2.1. Reagents

The reagents used for the purification and the development of the radioimmunoassay technique (RIA) were from Merck-VWR International or Sigma-Aldrich, Belgium (analytical grade), except where otherwise indicated. Bovine albumin used for the preparation of buffers was of RIA quality (Sigma). Polyethylene glycol 6000 was from Fluka. Radioactive iodine ( $^{125}\text{I}$ ) was from ICN laboratories ( $\text{Na}^{125}\text{I}$ , carrier-free, alkaline solution). The Sephadex gels, ACA 44 and activated 4B Sepharose, and the electrophoresis polyacrylamide gels (SDS-PAGE) were obtained from Pharmacia (Amersham-Bioscience). Normal rabbit serum and the sheep antiserum against rabbit gammaglobulins were delivered by the Laboratory of Hormonology (Centre d'Économie Rurale, Marloie, Belgium). Animal housing and immunisations were performed at the Laboratory of Hormonology (Centre d'Économie Rurale, Marloie, Belgium).

### 2.2. Protein measurement

The protein measurement was based on the registration of absorbance at 280 nm during the chromatographic steps of trypsin purification. Protein concentration was achieved by the spectrophotometric method of Folin Ciocalteu for the dialysed and concentrated extracts following purification.

### 2.3. Measurement of trypsin activity

During the purification, the presence of trypsin in the chromatographic fractions was determined by the measurement of its enzymatic activity using a low molecular weight chromogenic substrate [either

D-L-benzoyl-arginine-p-nitranilide (BAPNA) or carbobenzoxy-valyl-glycyl-arginine paranitranilide]. This technique is based on the proteolytic cleavage by trypsin of the chemical bond between arginine and para-nitroanilide, releasing para-nitroaniline, whose absorbance was monitored at 405 nm as previously described (kinetic measurement, 15 min at 25 °C, spectrophotometer Perkin Elmer lambda 5 or on microplates with the Multiscan Ascent reader Thermolab Systems) [19, 27, 44]. BAPNA was used (after trypsinogen activation) for the gel filtration chromatography fractions and carbobenzoxy-valyl-glycyl-arginine paranitranilide (more sensitive) for the affinity chromatography fractions. In our working conditions, we did not observe interference with other pancreatic enzymes like chymotrypsin, elastase, kallikrein, collagenase, lipase, amylase and carboxypeptidase [19].

### 2.4. Purification of equine trypsin

#### 2.4.1. Tissue extraction, $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysis

We followed the method previously described for the purification of human trypsin [4, 46]. All the extraction steps were conducted at +4 °C. The pancreas, from horses euthanised for severe limb affections and stored frozen (-70 °C), was extracted (three times) in  $\text{H}_2\text{SO}_4$  0.25 N, 0.1% Tween 20 (pH  $\geq$  2.5). The extract was centrifuged for 30 min, firstly at 1 500 g, and secondly at 20 000 g. The supernatant was treated with ammonium sulfate  $(\text{NH}_4)_2\text{SO}_4$  firstly at 20% saturation and secondly at 80% saturation. The final precipitate (obtained after centrifugation at 10 000 g, 30 min) was dialysed in  $\text{HCl}$   $10^{-3}$  M and concentrated.

#### 2.4.2. Chromatographic steps and electrophoresis

The proteins (dissolved in the elution buffer) were loaded on a gel filtration

column (Sephadex G75) and eluted with  $10^{-3}$  M HCl, 0.02 M  $\text{CaCl}_2$ , 0.2 M NaCl, pH 3.0. The trypsinogen-rich fractions were identified by trypsin activity measurement (BAPNA substrate) on a 100  $\mu\text{L}$  aliquot volume of each fraction (after trypsinogen activation by 1% enterokinase) pooled, dialysed and concentrated. Trypsinogen in the protein pool was then activated by 1% porcine enterokinase (0.01 M Tris-HCl buffer, pH 7.8, 20 mM  $\text{CaCl}_2$ , 0.05 M NaCl; +4 °C, 6 h) [10]. The last step was affinity chromatography on 4B Sepharose linked to aprotinin [30]. The affinity column was successively eluted with 0.1 M Tris-HCl buffer, NaCl 0.5 M, pH 8.0 (elimination of unbound proteins), 0.1 M Na-acetate buffer, NaCl 0.5 M, pH 4.5 (elimination of elastase and anionic trypsin) and 0.1 M Tris-glycine buffer,  $\text{CaCl}_2$  0.05 M, pH 2.5 to detach trypsin. The trypsin-rich fractions, identified by trypsin activity measurement (carbobenzoxyl-valyl-glycyl-arginine paranitranilide substrate) on a 50  $\mu\text{L}$  aliquot volume of each fraction, and adjusted to pH 4.0 with 0.05 M acetate buffer, were pooled, dialysed against acetate buffer and concentrated. The protein concentration was measured and the purity was estimated by acrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE 8-18) in non reducing/denaturing [40] and in reducing/denaturing conditions [51]. Equine trypsin for immunisation and RIA was kept in a solid form (aliquot fractions) at  $-70$  °C. Degradation studies were performed on trypsin solutions and on aliquot fractions of solid trypsin kept at  $-20$  °C or  $-70$  °C for several months.

## 2.5. Development of the radio-immunoassay

### 2.5.1. Immunisation

Two rabbits were immunised by an intradermic injection of 100  $\mu\text{g}$  of equine

trypsin (emulsified in 0.05 M phosphate buffer, 0.15 M NaCl, pH 7.4 supplemented with complete Freund adjuvant) followed by booster injections of 50  $\mu\text{g}$  every 15 days [7, 48]. Blood samples were collected 12 days after each booster injection. The serum was then analysed for its immunological reaction with equine trypsin by radial immunodiffusion and by binding to equine  $^{125}\text{I}$ -labelled trypsin (labelled antigen). The titer of the final blood sampling (rabbit exsanguination under anaesthesia) was defined as the highest antiserum dilution still binding 30% of the labelled antigen [7].

### 2.5.2. $^{125}\text{I}$ labelling

Equine trypsin (5  $\mu\text{g}$  in 5  $\mu\text{L}$  of 0.5 M phosphate buffer at pH 7.4) was achieved by chemical binding of  $^{125}\text{I}$  (1 mCi  $\text{Na}^{125}\text{I}$ , carrier-free, in an alkaline solution,) in the presence of 20  $\mu\text{g}$  of chloramine T [18, 30]. The reaction was stopped after 30 s by the addition of 20  $\mu\text{g}$  of sodium metabisulfite ( $\text{Na}_2\text{S}_2\text{O}_5$ ) and dilution with 0.05 M phosphate buffer, pH 7.4. Unbound  $^{125}\text{I}$  was eliminated by gel filtration chromatography on Sephadex G-25 eluted with 0.05 M phosphate buffer, 0.05%  $\text{NaN}_3$  and 0.5% bovine serum albumin. The fractions (1 mL) with the highest radioactivity ( $\gamma$  counter RIASTAR-Canberra-Packard) were pooled and further purified by gel filtration on ACA 44 (elution with the same phosphate buffer) to eliminate polymerised or degraded forms of the enzyme. The fractions with the highest radioactivity were pooled, kept at  $-70$  °C and used within 15 to 20 days. Before RIA incubation, the labelled antigen was diluted to  $\pm 40\,000$  cpm/100  $\mu\text{L}$  (tracer).

### 2.5.3. RIA procedure

The RIA method was a double-antibody technique (immunoprecipitation of the antigen-antibody complexes in the presence of sheep anti-rabbit-gammaglobulins)

with competition between the tracer and the TLI for a fixed amount of antibodies [20]. All the RIA reagents were diluted in the "incubation buffer": 0.05 M phosphate buffer, 0.05%  $\text{NaN}_3$  and 0.5% bovine serum albumin. The following solutions were left to incubate (various incubation times and temperatures were tested): (a) 100  $\mu\text{L}$  of the tracer, (b) 100  $\mu\text{L}$  of the successive dilutions of equine trypsin (from 1.25 to 250 ng/mL) or 100  $\mu\text{L}$  of the plasma to be tested, (c) 100  $\mu\text{L}$  of antiserum (1/6000 dilution). At the end of the incubation, 100  $\mu\text{L}$  0.1% normal rabbit serum and 1 mL of the precipitation solution (incubation buffer added with 60 g/L polyethylene glycol 6000, 200 mg/L of microcrystalline cellulose, 5 mL/L of Tween 20 and 0.5% sheep anti-rabbit gammaglobulin serum) were added and incubation was continued for 20 min at room temperature. Free and antibody-bound antigens were separated by centrifugation (2000 g, 20 min), and the precipitates were counted. The results are expressed as the ratio ( $B/B_0$ , expressed in %) between the amount of the tracer bound to the antibodies in the presence (B) and in the absence ( $B_0$ ) of equine trypsin. The  $B/B_0$  values calculated for the unknown samples are reported on a standard curve to obtain the TLI value in ng/mL [7].

The optimal conditions of RIA were achieved when the duration, the temperature of incubation and the final dilution of the antiserum permitted a 40% value for  $B_0$ , with a non-specific binding not exceeding 5%.

## 2.6. Characteristics of the RIA and application to equine plasma samples

### 2.6.1. Specificity, sensitivity, precision, accuracy, and reproducibility

The cross-reactivity of the antiserum was tested with equine albumin and the total protein contents of blood cells (iso-

lated leucocytes or erythrocytes used after homogenisation). The sensitivity was defined as the minimum amount of unlabelled trypsin that caused a reduction in the percent of the labelled antigen bound to the antibody, greater than twice the standard deviation of 10 determinations of  $B_0$ . The precision was estimated by the coefficient of variation calculated for 10 determinations of each standard trypsin concentration. The accuracy was estimated by the recovery of known amounts of trypsin added to equine plasma samples. The reproducibility was measured by the coefficient of variation calculated for at least five determinations of the same sample within or between assays [7].

### 2.6.2. Normal and pathological values

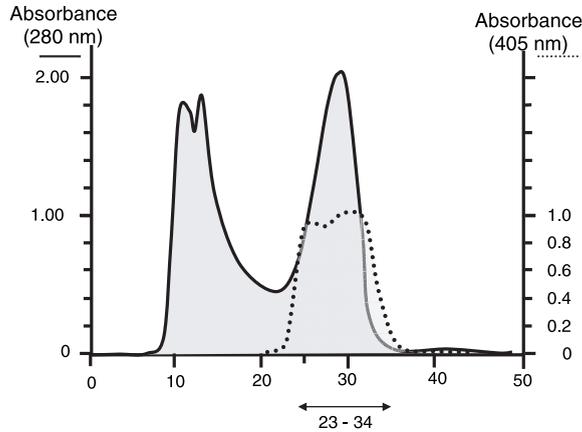
We established a reference range for TLI values in the plasma and serum of healthy horses ( $n = 20$ ), and applied the RIA to the plasma of horses suffering from acute intestinal obstruction ( $n = 10$ ). In these horses, TLI was assayed on admission (P1), during the surgery (P2) and the intensive care period about 24 hours after the end of surgery (P3). Another plasma sample (P4) was obtained when severe complications occurred or when the horse completely recovered a normal intestinal function (3–4 days after the surgery) except for the horses No. 9 and 10, which died early after the surgery.

## 3. RESULTS

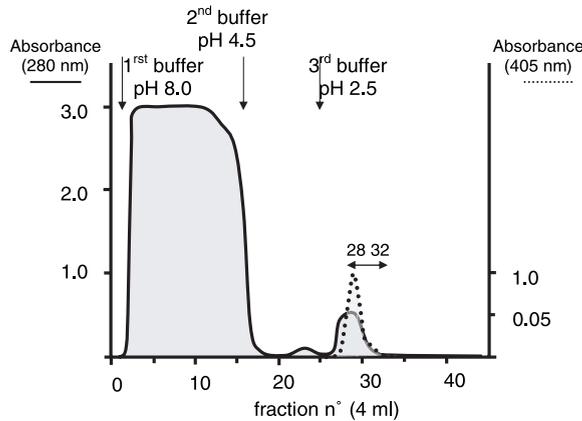
### 3.1. Isolation of equine trypsin

#### 3.1.1. Extraction and chromatography

After acid extraction of the pancreas, we obtained a precipitate containing 7 284 mg of proteins. Ammonium sulphate precipitations eliminated  $\pm 80\%$  of these proteins. Gel filtration chromatography on Sephadex G75 eliminated the remaining proteins



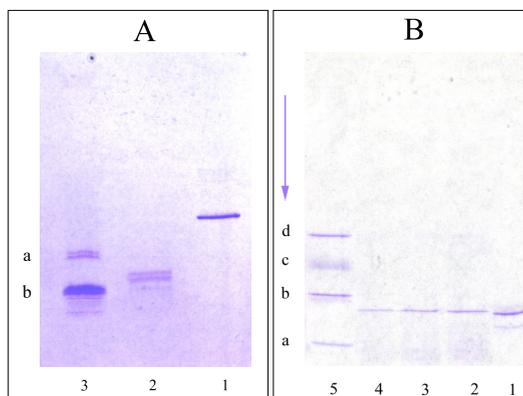
**Figure 1.** Gel filtration chromatography (Sephadex G-75) of the equine pancreas extract. Left ordinate: — absorbance of the proteins at 280 nm; right ordinate: ..... trypsin enzymatic activity (measured on a 100  $\mu$ L aliquot of each eluted fraction after trypsinogen activation with enterokinase).  $\leftrightarrow$  pool of trypsinogen-rich fractions.



**Figure 2.** Last step of equine trypsin purification: affinity chromatography on sepharose-aprotinin. Left ordinate: — absorbance of the proteins at 280 nm; ..... right ordinate: trypsin enzymatic activity (measured on a 50  $\mu$ L aliquot of each eluted fraction).  $\leftrightarrow$  pool of trypsin-rich fractions. Arrows indicate the changes of the elution buffer.

with molecular weight  $>80$  kDa, and trypsinogen was detected in the second peak (Fig. 1). The pool of trypsinogen-rich fractions (fractions 23 to 34) was activated with enterokinase to release trypsin, and used for affinity chromatography. This second chromatography step eliminated the remaining pancreatic proteins (eluted

in the first and second peaks). The trypsin rich fractions (fractions 28 to 32) were detected in the last peak (Fig. 2). From 122 g of equine pancreas, 18 mg of lyophilised proteins with a trypsin enzymatic activity  $>90\%$  were obtained at the end of the purification procedure (extraction rate of 0.25%).



**Figure 3.** Analysis of purified equine trypsin by SDS polyacrylamide (8-18) gel electrophoresis. Part A: Electrophoresis of equine trypsinogen obtained after gel filtration chromatography. Lane 1: bovine albumin; lane 2: bovine trypsin/trypsinogen; lane 3: equine trypsinogen after gel filtration chromatography: bands a are trypsinogen and band b represents proteins with molecular weight around 18 kDa (no enzymatic activity).

Part B: Electrophoresis of equine trypsin obtained after affinity chromatography. Lane 5: molecular mass markers (d: bovine albumin, 67 kDa; c: ovalbumin, 43 kDa; b: carbonic anhydrase, 30 kDa; a: aprotinin, 12 kDa). Lanes 4 and 3: equine trypsin (40 µg and 20 µg) after affinity chromatography (no reduction and no denaturation). Lane 2: same as lane 3 after reduction and denaturation). Lane 1: bovine trypsin/trypsinogen (40 µg). Arrow indicates the migration direction (part A and B).

### 3.1.2. SDS-PAGE analysis

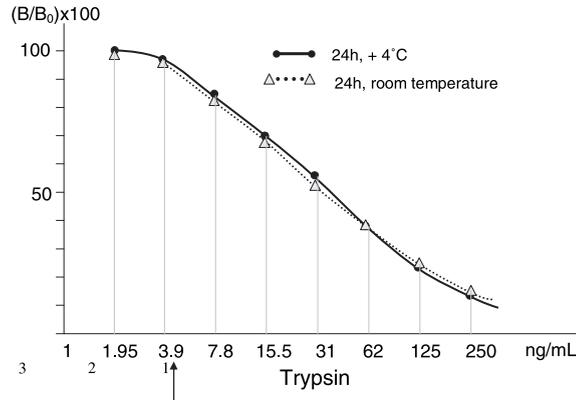
SDS-PAGE performed on the pool of trypsinogen-rich fractions obtained after gel filtration chromatography, showed two bands at  $\pm 27$  and  $\pm 26$  kDa, a large band at  $\pm 18$  kDa and a lot of low molecular weight bands (Fig. 3, part A). The 27 and  $\pm 26$  kDa bands slowly developed a positive enzymatic response in the presence of carbobenzoxy-valyl-glycyl-arginine para-nitranilide. In reducing and denaturing conditions, no changes were observed in the electrophoretic profile.

The final preparation showed 96% purity as estimated by gel electrophoresis (densitometry on the stained gel) (Fig. 3, part B). The SDS-polyacrylamide gel electrophoresis in non reducing/denaturing conditions showed one band with a molecular weight of  $\pm 27$  kDa. Enzymatic activity

was detected on this 27 kDa band. The electrophoresis of the enzyme after reducing/denaturing treatment did not show any additional band suggesting that the enzyme was monomeric.

### 3.1.3. Degradation analysis

When trypsin was kept in solution at  $-20$  °C for three months, new electrophoretic bands ( $<10$  kDa and mainly around 3 kDa) were observed (without enzymatic activity), attributed to small trypsin degradation fragments. Lyophilised trypsin kept in the same conditions did not show degradation bands. At  $-70$  °C after 6 months, no degradation was observed for equine trypsin powder but was present for trypsin solutions: electrophoresis showed two major fragments ( $\pm 15$  and 12 kDa) that still retained enzymatic activity.



**Figure 4.** Standard curves of RIA for equine TLI. Two incubation conditions were compared: 24 h at +4 °C or 24 h at room temperature. The reference molecule was undegraded equine trypsin. Arrow indicates the limit of sensibility of the RIA (4 ng/mL).

### 3.1.4. Main characteristics of the final equine trypsin

The enzymatic activity (with carboben-zoxyl-valyl-glycyl-arginine paranitranilide used as the substrate) was  $\text{Ca}^{2+}$ -dependent with optimal concentrations around 20 mM and was maximal at pH 8.0. It was also inhibited by aprotinin and the soybean trypsin inhibitor (molecular ratio inhibitor/trypsin 2/1), azide and benzamidine (molecular ratio inhibitor/trypsin 500/1). Equine trypsin activity on low molecular weight substrates was reduced by albumin (0.5%) and equine plasma.

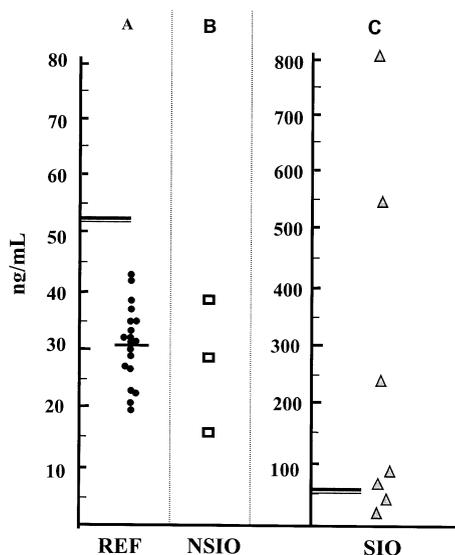
### 3.2. Labelling and immunisation

Lyophilised batches of equine trypsin, without degradation, were used for rabbit immunisation. From two rabbits, we obtained two antisera that were used at an initial dilution of 1/6 000 with a maximal  $B_0$  value of 40% achieved after 4 h of incubation, either at +4 °C or room temperature (18–20 °C), remaining stable for 36 h of incubation, with non-specific binding between 2 and 4%. The chloramine T technique of labelling followed by gel filtration chromatography yielded a labelled anti-

gen, uncontaminated by free iodine and degraded trypsin forms. The tracer was used at a dilution of 40 000 cpm/100  $\mu\text{L}$  and remained immunoreactive until 20 days after labelling.

### 3.3. RIA conditions

The RIA conditions were the following: total activity adjusted to 40 000 cpm for 100  $\mu\text{L}$  of labelled antigen solution, antiserum at a dilution of 1/6 000 with an incubation time of 16–18 h (overnight), representing the easiest laboratory conditions. Figure 4 shows that the lower detection limit of the RIA was 4 ng trypsin or TLI/mL and that the upper limit was 200 ng/mL. The reference curve was obtained at room temperature and superimposed on the curve obtained at +4 °C (Fig. 4). From these results, we chose an overnight incubation at +4 °C for easy working conditions during our subsequent assays. The coefficient of variation calculated from 10 determinations of each point of the reference curve was always lower than 4% for equine trypsin concentrations ranging from 10 to 150 ng/mL, indicating an excellent reproducibility. The coefficient of variation intra- and interassay ( $n = 10$ ) for



**Figure 5.** Individual values of plasma TLI (ng/mL). A: in 20 healthy horses (REF), with mean reference value (—) and upper limit of normal (===). B: maximal value measured in 3 horses with non strangulating intestinal obstruction (NSIO). C: maximal value measured in 7 horses with strangulation obstruction (SIO). === Upper limit of normal.

unknown plasma samples never exceeded 6% (in the linear part of the curve). The recovery of known amounts of equine trypsin (from 10 to 100 ng/mL) added to serum samples was  $\pm 90\%$ , indicated a high level of accuracy. There was no cross-reaction with albumin or with the intracellular content of equine leucocytes or erythrocytes.

### 3.4. Application to plasma samples and establishment of a reference range

The values measured in the plasma of healthy horses ( $n = 20$ ) are represented in Figure 5, with a mean value of  $30.01 \pm 6.84$  ng/mL. The mean value + 3 SD (50.52 ng/mL) was chosen for the upper confidence limit with a probability of error of  $p < 0.01$ . In serum samples ( $n = 20$ , the same horses as for the plasma measure-

ment), the normal value was  $32.05 \pm 6$  ng/mL, not statistically different from the plasma values.

### 3.5. Preliminary results in acute intestinal obstruction

Three horses showed non-strangulating lesions without post-operative complications. Their TLI values always remained within the normal limits (horses No. 1, 2 and 3; Tab. I). Seven horses were presented with strangulating small or large intestine obstruction. Five of them, three on admission (horses No. 7, 9 and 10; Tab. I), one after surgery (horse No. 6) and one during the post-operative phase (horse No. 4; Tab. I) did show an increase in the TLI values above the normal physiological values. In particular, two horses (No. 9 and 10) with severe intestinal lesions that did not survive had very high levels in each plasma sample (Tab. I).

In Figure 5 (parts B and C), we compared the highest individual value measured for each horse with acute intestinal obstruction to the individual values of the reference horses (part A).

## 4. DISCUSSION

### 4.1. Purification of equine trypsin

Apart from a short abstract [21], the purification of equine trypsin has never been described extensively, in contrast to the enzyme in humans, bovines and many other species. Therefore, we designed a purification method for equine trypsin based on our experience with human trypsin and on previously reported methods of trypsin purification [4, 25, 46]. The first purification steps were conducted in an acid milieu thus permitting the limitation of the autoactivation of trypsinogen and avoidance of the addition of enzymatic inhibitors during the purification. They eliminated  $\pm 80\%$  of the total protein

**Table I.** Trypsin-like immunoreactivity (TLI) in horses with intestinal obstruction ( $n = 10$ ).

Horse	Intestinal pathology	Issue	TLI (ng/mL)			
			admission (P1)	surgery (P2)	24 h after surgery (P3)	3–4 days after surgery (P4)
1*	Ileum impaction	S	17	28	13	7
2*	Large colon displacement	S	13	14	10	11
3*	Large colon displacement	S	31	38	32	26
4**	Ileal incarceration in epiploic foramen	S	19	50	19	96
5**	Ileal incarceration in epiploic foramen	S	17	14	17	7
6**	Large colon torsion	S	29	71	22	19
7**	Large colon torsion	S	230	98	34	20
8**	Jejunal incarceration in epiploic foramen	NS	31	20	7	10
9**	Volvulus of the jejunum	NS	800	180	178	§
10**	Large colon torsion	NS	500	546	210	§

\* Non strangulating obstruction; \*\* strangulation obstruction.

S: survivor; NS: non survivor. §: no value (death shortly after surgery).

content, and gel filtration chromatography (G-75) removed 50% of the remaining proteins with a molecular weight  $\geq 80$  kDa. At this point, we isolated trypsinogen, but a low trypsin activity could be detected in the fractions corresponding to the last part of the first protein peak eluted from the gel filtration column, this being attributed to the presence of unstable trypsin complexes with pancreatic high molecular weight inhibitors. From electrophoresis, we concluded that the pool of trypsinogen-rich fractions contained quantities of contaminating proteins of low molecular weight, requiring a new purification step. No explanation can be given for the presence of two trypsinogen bands: their slow kinetics of autoactivation indicated the absence of trypsin and suggested the existence of two forms of equine trypsinogens, conditions similar to those reported for human trypsinogen [4, 10].

This hypothesis has to be confirmed by complementary studies.

Affinity chromatography yielded a pure monomeric protein, positive for enzymatic detection, and not contaminated by elastase or chymotrypsin (their enzymatic activities were not detectable using specific low molecular weight substrates).

Molecular weight was estimated around 27 kDa. There is little published data on the equine trypsin: Harris and Hofmann [21] isolated the equine trypsinogen with a molecular weight of 25.6 kDa. Walsh [49] (reporting complementary data obtained as a personal communication from Hofmann) attributed a total of 226 to 229 amino acids (201 for human trypsinogen) to equine trypsinogen, with a N-terminal sequence of 8 amino acids, which is split to produce active trypsin. By electrophoresis, we found similar results, but molecular weight

determinations by electrophoresis are not very precise, and the exact molecular weight of equine trypsin will only be known after complete sequencing.

In our working conditions, the yield of extraction was low, mainly due to the incomplete activation of trypsinogen by enterokinase. The remaining trypsinogen was detected after activation in the peak on unbound proteins and eluted with the first buffer (pH 8.0) on the affinity column.

The global scheme of purification was similar to that used for the human enzyme [4, 30, 46], and was rapid, but had a low efficiency due to the incomplete activation of trypsinogen into trypsin by enterokinase. This low activation could be attributed to the pH of the milieu (pH 7.8) and to the time and temperature of activation (6 h at +4 °C). This pH value was chosen because it allowed an easy pH adjustment necessary for the next step of purification (pH 8.0). The time and the temperature were chosen to limit the degradation of trypsin during the activation. For the next purification, activation will be performed at pH 6.0 a value near the physiological value for enterokinase activity. We did not choose the autoactivation of trypsinogen at pH 8.0 in the presence of  $\text{Ca}^{2+}$  because this technique needed a longer reaction time at room temperature, with an increased risk of degradation. Moreover, we could not exclude the possibility that, after the gel filtration chromatography step, pancreatic inhibitors remained in the pool of proteins used for activation. These inhibitors would block free trypsin already present in the pool, slowing the autoactivation and increasing the degradation. Finally, we had doubts about the correct  $\text{Ca}^{2+}$  concentration to be used, since previous studies on human and bovine trypsinogens demonstrated major differences, and that excessive concentrations of this ion were inhibitors on human trypsinogen activation [4].

Equine trypsin reacted like human or bovine trypsin with classical inhibitors of serine proteases and was inhibited by

plasma [9, 10, 37]. Its inhibition by  $\alpha_2$ -M or  $\alpha_1$ -PI remained unknown on account of the need for purification of these equine anti-proteases prior to testing. The role of equine plasma can be attributed to trypsin trapping by  $\alpha_2$ -M, slowing the enzymatic activity on the substrate. Albumin has never been described as an inhibitor, but as a protector of trypsin [30]. Its "in vitro effect" on equine trypsin can be attributed to a "competitive" action of albumin slowing the reaction of trypsin with the carbobenzoxy-valyl-glycyl-arginine paranitride substrate.

The equine enzyme seemed to be fragile in solution, as shown by the presence of small bands of degradation observed by electrophoresis. The degradation remained rapid, even when the enzyme was stored frozen at -70 °C. But, the final preparation of equine trypsin was lyophilised and kept in this solid form at -70 °C. Trypsin solutions were prepared for immediate use (before labelling and before injection for rabbit immunisation); it thus seemed unlikely that they contained seriously degraded forms.

#### 4.2. Establishment of the RIA

We applied the classical  $^{125}\text{I}$  labelling technique with chloramine T, as previously used for many proteins [18] and for human trypsin [30] and obtained a labelled antigen that was stable for 20 days in phosphate buffer supplemented with bovine serum albumin. We obtained two antisera that were used at a dilution of 1/6 000 (yielding 40% of binding of the labelled antigen). The equilibrium of the antibody-antigen reaction was reached after 4 h of incubation at +4 °C or room temperature and did not vary until 36 h of incubation. Thus, the conditions of the assay, 16–18 h of incubation at +4 °C, were chosen to facilitate the work: overnight incubation allowed an easy planning of the assays, and +4 °C provided a fixed temperature independent of laboratory temperature variations.

The reference curve showed a good sensitivity of the RIA with a detection threshold of 4 ng/mL and a superior limit of 200 ng/mL, comparable to the reference curves and the sensitivity limits (from 0.6 to 10 ng/mL) previously established for the RIA in other species [2, 11, 16, 30, 43, 45, 52]. The intra- and interassay variation coefficients were low, never exceeding 6% for trypsin values in the linear part of the reference curve, indicating that the RIA was precise and reproducible. The assay was specific because the antibodies did not recognise albumin or the global content of equine leukocytes or erythrocytes. There was no cross-reaction with human or bovine trypsin/trypsinogen. A high degree of accuracy was achieved, since 90% of the added amounts of trypsin were detected by the assay.

#### 4.3. Estimation of normal and pathological values

The mean value of TLI in the horse ( $30.01 \pm 6.84$  ng/mL) was similar to the mean value observed in humans ( $26-30 \pm 12$  ng/mL) [2, 6, 11, 16, 24, 30] and other species [14, 43, 52]. The superior limit of normal values was 50 ng/mL (corresponding to the mean value + 3 SD,  $p < 0.01$ ). In the absence of previously described equine TLI RIA, no comparison could be made, nevertheless, the value was similar to the human maximal physiological value of 70 ng/mL [25]. The sampling conditions (immediate versus delayed centrifugation), the use of serum or anticoagulated blood, and the type of anticoagulant, did not influence the results of TLI in the horse. This conformed to observations in humans but should be examined on a larger number of horses. Within the samples of horses suffering from intestinal obstruction, only those cases with strangulation obstruction showed high TLI concentrations, this increase being observed in horses suffering from small as well as large intestine obstruction. In a previous study [19], we already demonstrated an increase in the

plasma active trypsin concentration in horses suffering from intestinal obstruction and shock, and the assay of TLI we report in this study was complementary to the assay of trypsin activity. Further studies using the two assay methods should be led in order to evaluate the degree of pancreatic implication in equine acute abdominal disease.

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