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# Bovine Pepsinogen A: Isolation and Partial Characterization of Isoforms with High Activity

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**Abstract:** The goal of this study was to purify bovine pepsinogen by a simple method allowing the preparation of large amount of pure protein. The purified protein and antisera are needed to develop diagnostic methods for further investigations in animals susceptible of gastric disorders or helminthosis. Pepsinogen isoforms were separated from extracts of bovine fundic mucosa by ammonium sulfate precipitations and chromatography on DEAE and hydroxyapatite. The isoforms showed a high activity in indirect proteolytic assay. Sequence analysis gave the following amino acid sequence SVVKIPLVKK for fraction 1, 2 and SVVKIPLVKKKSLRQNLIENGKLKE for fraction 3. The Mass spectrometry revealed isoforms with different masses from 39,864 to 40,181 Da. The estimated organic phosphate content ranged from 0.98 to 3.9 moles of phosphate per molecule. The protocol, with few steps, gave consequent quantities of pure and active protein available for further studies including the development of RIA and ELISA as diagnostic tools in gastrointestinal diseases.

**Key words:** Bovine Pepsinogen, Purification, mass spectrometry, N-terminal sequence, phosphorylation

## INTRODUCTION

Gastrointestinal parasites are among several factors that contribute to problems in cattle husbandry by inducing economic losses. Some parasites like *Ostertagia* or *Haemonchus* damage the abomasa walls leading to a greater permeability to macromolecules like pepsinogen that may reach the blood stream. As early as 1965, Anderson *et al.*[1] demonstrated the value of the plasma pepsinogen test as a diagnostic aid for bovine ostertagiasis. Further experimental studies have confirmed the relation between the level or duration of infestation and serum pepsinogen concentration[2-4]. Since, several studies including pepsinogen determination and parasitology have been conducted in the monitoring of cattle husbandry. It has been reported that during the first grazing season, high values of serum pepsinogen correlates with the appearance of gastro-intestinal parasites. However, various diagnostic thresholds were reported on infected animals and made the comparison difficult. Kerboeuf[5] reported for asymptomatic animals values of 300-600 mU (milli-units of tyrosine) and 2000-3000 mU for animals with damaged abomasa. In 1989, Hilderson *et al.*[6] gave a limit of 5000 mU in the case of *Ostertagia* infestation whereas Berghen *et al.*[7] found rates of 3500 mU in infected animals. A study of Vercruysse and Claerbout stated that at housing untreated calves with sub-clinical infections have a blood pepsinogen level in the range of 2000-4100 mU, while calves presenting clinical infections had values between 3700 and 6300 mU. The measurement of pepsinogen concentrations in blood samples using different methods revealed a great variability in values[8]. In fact, the first described method[9] consisted in measuring the proteolytic activity of the pepsin present in the serum on a substrate in acidic condition. Since, many variations of the basic method have been published[5,10,11]. Until recently the published techniques are still enzymatic[12]. Despite the great interest of measurement of pepsinogen in blood of cattle, only few alternative techniques were described in the scientific literature. Except the description of a radial immunoassay by Thode-Jensen[13] and an ELISA by Gomes *et al.*[14], we are aware of any study dealing for the development of Radioimmunoassay (RIA). Nevertheless, since 1974, Samloff and Liebman[15] reported that, in humans, the enzymatic methods for pepsinogen proportioning measured the total activity of all the zymogens present in blood, these methods were then progressively forsaken and radioimmunological methods were developed[16]. The RIA exhibits high specificity permitting to differentiate different type of aspartic proteases. A recent study in porcine species by

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comparison of radioimmunoassay and enzymatic method has been conducted in our laboratory[17]. No difference of concentration was found between pigs with ulcer and pigs with parakeratosis in the enzymatic method, whereas the RIA differentiates the two groups. This study clearly demonstrates the additional enzymatic effect of chymosin which is found in high concentration in prenatal or young piglet sera. The conclusion was that the enzymatic method measures the cumulative concentration of different proteases, while radioimmunoassay is able to quantify a specific protein (eg pepsinogen A, pepsinogen C, chymosin or cathepsin...). All together, these observations in human and animals species indicate clearly a lack of the enzymatic methods specificity (in which all acid proteases are more or less active) and point out the interest of specific methods for each different type of aspartic protease (chymosin, cathepsin D and pepsinogen of different type...).

Human pepsinogen A and C, porcine pepsinogen A and bovine chymosin B are the most important aspartic proteases commercially available, bovine pepsinogen is not yet marketed. Gastric aspartic proteases were found to be colocalized in the same secretory granules of cells of fundic mucosa[18]. They are synthesized as inactive precursors known as zymogens and stored in the chief cells of the mucosa. For their purification, abomasa are extracted and protein are fractionated using salt precipitation and chromatography.

The high susceptibility of pepsinogen to denaturation is one of the reasons that could explain the difficulties in the development of purification protocols and the availability of pure proteins for laboratory animals immunization. Different methods of purification involving several steps of conventional chromatography have been developed[19,20]. Meitner and Kassell[21] isolated different isoforms of the zymogen corresponding to bovine pepsinogen A, which differ only in their phosphate content. Gomes *et al.*[14] obtained an homogeneous preparation of bovine pepsinogen after a DEAE cellulose and Mono Q columns with estimated molecular weight of 46kDa. Bovine pepsinogenC or progastricsin has been first isolated by Antonini and Ribadeau-Dumas[22] who reported some similitude with prochymosin in electrophoretic mobility and the optimum pH range (2.7-2.9) of proteolytic activity towards haemoglobin. They reported also that leucine is the N-terminal amino acid for this zymogen. Bovine pepsinogen C was identified also by Eckersall *et al.*[23] who reported that it was eluted earlier from an anion-exchange column and is 30 times less abundant than pepsinogen A.

Despite the relevance of clinical studies in relation to gastric diseases (helminthosis, abomasa displacement or ulcers), no specific assay was developed to investigate specifically pepsinogen and related proteases in bovine serum. It is a fact that the principle of the well known proteolytic methods consists to acidifying sera samples in order to convert zymogens into active enzymes; in addition, it is known that gastric proteases show a similarity in their activation to active enzymes when the optimum pH is lower than 4 (range 2 and 3.5)[24]. So, as protease activity depends essentially on pH and on the nature of the substrate used, the enzymatic methods are thus not specific for a determined gastric protease but measures the cumulative concentration of different proteases, while radioimmunoassay is able to quantify a specific molecule. With regard to the development of improved radioimmunoassay for blood pepsinogen measurement, the first step was to produce sufficient amount of the pure enzyme for specific antisera production.

Thus, the current investigation was designed to develop a protocol for bovine pepsinogen purification giving a pure preparation of the molecule for the specific antisera production. Both pure protein and specific antisera are needed for radioimmunoassay development in blood. This study, presented the results of a purification procedure and some characteristics of the purified protein.

## MATERIALS AND METHODS

**Protein assay:** For each step, Total Protein content (TP) of all fractions was determined by the method of Lowry *et al.*[25] using bovine serum albumin as standard (BSA: Fraction V Fatty Acid Free; Sigma St Louis).

**Enzymatic activity:** The indirect pepsinogen evaluation method described by Dorny and Vercruysse[11] was used with modifications[17]. Potential pepsin activity of pepsinogen from all fractions was determined after 24 h incubation at pH 2.0, the temperature of incubation was 37°C (with 2% BSA solution as substrate). The same test was also used to measure the proteolytic activity of the preparations and to compare with the bovine chymosin, human and porcine pepsinogen commercially available.

**Protein extraction:** Bovine abomasae were collected from the slaughterhouse. The fundic area was cut out and washed thoroughly to prevent activation of the zymogen by the highly acidic gastric juice; the mucosa was separated and frozen at -20°C for storage until used. The mucosa was thawed, minced and homogenized in 10 mM sodium phosphate buffer pH 7.3. The homogenate was centrifuged for 1 h at 18,000 g. The pellet obtained was discarded and the supernatant was subjected to Ammonium Sulfate (AS) fractionation.

**Ammonium sulfate fractionation:** Ammonium sulfate (Merck KGaA 64271 Darmstadt) was slowly added to the stirred supernatant to obtain 20% saturation. After 2 h, the solution was centrifuged during 1 h at 18,000 g. Supernatant was again subjected to 40% of ammonium sulfate saturation and centrifuged. The pellet was resuspended with 10 mM sodium phosphate buffer pH 7.3. The supernatant was precipitated to obtain 70% AS saturation. After 1 h centrifugation at 18,000g, the 70% pellet was resuspended with 10 mM sodium phosphate buffer at pH 7.3. The resuspended 40 and 70% ammonium sulfate precipitation pellets were extensively dialysed against 200 mM sodium phosphate buffer pH 7.0. All precipitation steps were carried out at +4°C.

**Purification by chromatography:** The Fast-Performance Liquid Chromatography (FPLC) was carried at room temperature.

**DEAE ceramic column:** The fraction precipitated by ammonium sulfate with the highest proteolytic activity was loaded on a Diethylaminoethyl (DEAE) ceramic column (9 x2 cm, Biosepra) pre-equilibrated overnight with 20 mM sodium phosphate buffer pH 7.0, then eluted by step with 0.05-0.1-0.2-0.5 and 1M NaCl, the flow rate was 1 mLmin$^{-1}$. The elution was monitored by UV absorption at 280 nm and 3 mLtube eluted fractions were collected and pooled according to the optical density. The active fraction was then loaded on a hydroxyapatite column.

**Hydroxyapatite (HT) column:** Hydrated hydroxyapatite gel was purchased from Bio-Rad (Bio-gel HT gel, Bio-Rad Laboratories, CA). The gel was resuspended as recommended by the manufacturer and the column (1.6x20 cm) was equilibrated with 20 mM phosphate buffer at pH 7.3. The flow rate was 0.75 mL min$^{-1}$ and the elution was monitored at 280 nm. Proteins were eluted with sodium phosphate gradient. The fraction eluted on DEAE ceramic with the highest proteolytic activity was loaded on the HT column and fractions of 1.5 mL/tube were recovered. After elution, fractions were dialysed against 20 mM sodium phosphate buffer pH 7.5, aliquoted and kept frozen at -20°C.

**Biochemical characterization**

**Electrophoresis:** One dimensional gel electrophoresis (SDS-PAGE 1%) was carried out on a vertical-cell system essentially as described by Laemmli[26]. Purified fractions were loaded on stacking gel of 4% polyacrylamide then separated on 12% polyacrylamide gel in the presence of SDS. Standards with following molecular weight were run simultaneously: Phosphorylase b (97 kDa), Albumin (66 kDa), Ovalbumin (45 kDa), Carbonic Anhydrase (30kDa), Trypsin Inhibitor (20.1 kDa) and α-Lactalbumin (14.4 kDa) (Protein mixture of Amersham Biosciences). Slab gels were run at 200 volts during 30 min of migration. To visualise separated bands at the end of migration, the gel was transferred into a solution of coloration (acetic acid-methanol-water 10:40:50; v/v/v) with Coomassie blue (0,025%) (R-250, Merck, Darmstadt) during 1h, and into a solution of destainer overnight (acetic acid-methanol-water 10:40:50; v/v/v). At each step of the purification, the collected peaks were examined by one dimensional gel electrophoresis and ten micrograms of proteins were loaded per well. To identify the proteins, the pure fractions were subjected to N-terminal sequence analysis and Mass Spectrometry.

**Amino acid sequence analysis:** Purified proteins were subjected to automatised Edman degradation about 400picomoles of purified protein on a pulsed liquid-phase protein sequencer (Procise 492 Applied Biosystems, Foster City, CA). The complex fraction was subjected to a transfer on Polyvinylidene Difluoride (PVDF) membrane and the major band was excised from the dried PVDF membrane before analysis. The standard amount was 10picomoles, the sampling rate was 4.0Hertz and detector scale was 1.000 AUFS. Amino-acid sequences obtained were compared to other protein sequences from EMBL and SWISSPROT data bank. The comparison was done with FASTA3.

**Mass Spectrometry and phosphate evaluation:** Negative ions were used for the spectrometry because of the presence of few basic amino acids in the molecule, only pure fractions were analysed on 1.2 Micromass Q-TOF Ultima (Micromass UK Limited). The error range of the apparatus was from 2 to 8 Da. The phosphate determination was based on the atomic mass of the ($H_3PO_4$) group. After binding on the OH group of the molecule, the phosphate group loses one atom of hydrogen and one atom of oxygen and became ($H_2PO_3$) with a molar mass of 80 Da. The total amount of the phosphate (mole per mole of protein) was then estimated.

## RESULTS

**Extraction and fractionation:** The starting material was 1,686 g of the mucosa. After ammonium sulfate fractionation, the highest proteolytic activity was found in the 40-70% precipitate, suggesting that bovine pepsinogen A molecules precipitated between 40 and 70% ammonium sulfate saturation.

**Chromatography on DEAE-ceramic:** An amount of 562 mg (total protein) of the 40-70% ammonium sulfate saturation was applied to the column. Stepwise elution

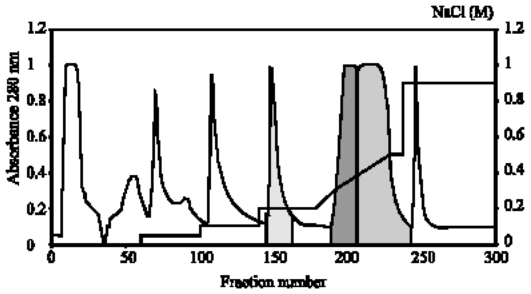


Fig. 1: FPLC chromatography on DEAE-ceramic. The column was 9 x 2 cm; equilibrated in 20 mM sodium phosphate buffer pH 7.0. Stepwise elution was performed with 0.05, 0.1, 0.2 linear between 0.2 and 0.5, and 1M NaCl, indicated here by continuous stairs line. Fractions (3 mL/tube) were collected at a flow rateof 1 mL/min. Black areas indicate the locations showing the highest proteolytic activity

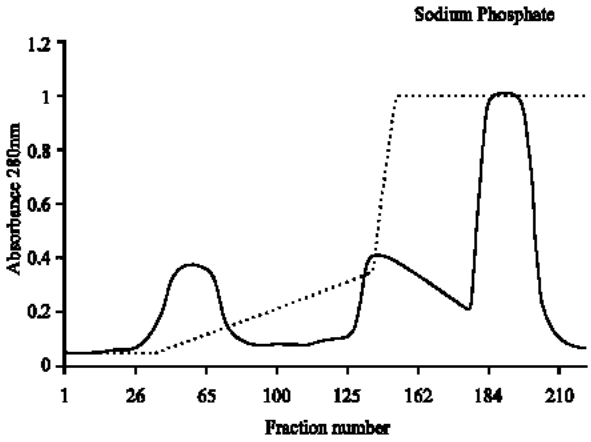


Fig. 2: FPLC chromatography on hydroxyapatite. The column was 1.6 x 20 cm; equilibrated in 20 mM sodium phosphate buffer pH 7.3, discontinuous stairs line indicate elution gradient. Fractions (1.5 mL) were collected at a flow rateof 0.75mL/min

was performed with 0.05, 0.1, 0.2 linear between 0.2 and 0.5 and 1M NaCl. The elution pattern is shown in (Fig 1.) Several peaks were separately collected and extensively dialysed against 20 mM sodium phosphate buffer pH 7.3 before being frozen at -20°C. The majority of reactive proteins were eluted at 300 mM. The isolation was then continued with this fraction.

**Chromatography on Hydroxyapatite (HT):** An amount of 34 mg (total protein) of the 300 mM peak collected from the DEAE ceramic column was loaded on the HT column. The elution pattern of the HT column was shown in (Fig .2). Three peaks were separately collected and extensively dialysed against 20 mM sodium phosphate buffer pH 7.5.

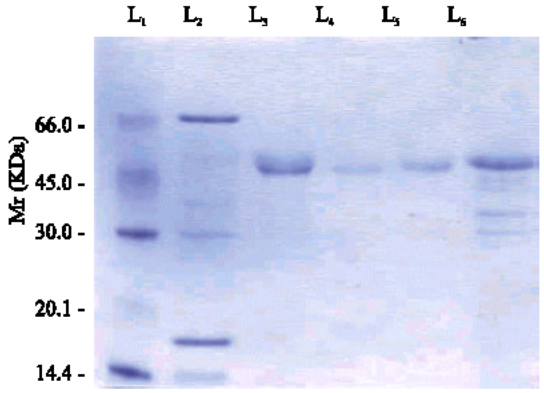


Fig. 3: Electrophoresis pattern of purified proteins at different steps purification. The collected fractions were analysed on a 12% polyacrylamide SDS gel. Line1: MW standard; $L_2$: 40-70% AS saturation; $L_3$: fraction 300 mM of DEAE ceramic; $L_4$: fraction 1 of hydroxyapatite column; $L_5$: fraction 2 of hydroxyapatite column; $L_6$: fraction 3 of hydroxyapatite column

The Three consecutive steps of the purification were shown in Table 1.

**One dimensional SDS-PAGE:** The collected peaks were examined by SDS-PAGE carried out on a vertical-cell system. Ten micrograms of proteins of each were loaded on stacking gel. (Fig .3) shows the migration pattern of successive purified fractions (after ammonium sulfate fractionation, after DEAE column and the 3 peaks collected after HT column). The purification level of the fractions could be easily assessed. The ammonium sulfate fraction showed a very complex banding while the DEAE fraction presented a complex banding with one major band between 40 and 50 kDa and some minor bands with great mobility. The SDS-PAGE analysis revealed, after HT column, three fractions, two simple single banding pattern around 45 kDa (fraction 30-76 ($L_4$) and fraction 124-180 ($L_5$)) and one complex with a major band around 45kDa and few minor bands with a greater mobility (fraction 181-200 ($L_6$).

Table 1: Total protein, total activity and specific activity are shown for each step of the purification. The recovered activity were calculated and presented in the last column

| Steps | Total protein (mg) | Total activity (U.tyr) | Specific activity U. tyr $mg^{-1}$ | Recovery (G% activity) |
|---|---|---|---|---|
| 70% AS saturation | 562 | 247.84 | 0.441 | 100 |
| DEAE (300 mM) | 34 | 197.2 | 5.8 | 79.56 |
| Hydroxyapatite: | | | | |
| Peak 1 | 10.03 | 51.70 | 5.15 | 20.86 |
| Peak 2 | 11.00 | 81.95 | 7.45 | 33.06 |
| Peak 3 | 12.00 | 81.76 | 6.81 | 32.98 |

Table 2: Organic phosphate content of bovine pepsinogen A on the basis of atom weight of phosphate group

| Zymogens | Pepsinogen A-1 | | | Pepsinogen A-2 | | |
|---|---|---|---|---|---|---|
| Isozymogens | a | b | c | a | b | c |
| Molecular Mass (Da) | 39864.98 | 39944.88 | 40024.73 | 40104.70 | 40142.93 | 40181.39 |
| Theorical phosphate residues (mol/mol) | 0 | 0.98 | 1.97 | 2.95 | 3.43 | 3.90 |

**Sequence analysis:** The proteins of the three peaks of the HT column were subjected to automatised Edman degradation to determine the N-terminal amino sequence. From the first and the second purified fractions, 10 residues were sequenced and 25 from the third fraction. The following amino acids were identified:

Fraction 1- SVVKI PLVKK
Fraction 2 - SVVKI PLVKK
Fraction 3 (major band)- SVVKI PLVKK KSLRQ NLIEN GKLKE

The three sequences showed 100% identity over 10 amino acids and the 25 amino acids of fraction 3 are totally identical to the 25 amino acids of the $NH_2$-terminal sequence of bovine pepsinogen A present in Swiss-prot data bank (accession number: P00792) or in GENBANK (accession number: AY330769).

The sequence was compared to other bovine protein sequences from EMBL and Swiss-prot data bank. The comparison was done with FASTA3 programme. Total identity (100%) was found with the sequence of bovine pepsinogen A (N-terminal amino sequence and sequence from cDNA)[27,28].

**Mass spectrometry and phosphate content:** The pure fractions (fraction 1 and 2) were analysed using negative ions because of the presence of few basic amino acids in the molecule. The MaxEnt processed data revealed three components with different molecular masses in each fraction. In fraction 1 (bPgA-1), the first isoform gave a molecular mass (MM) of 39,864.98 Da (bPgA-1a) and it weighs 79.9Da lower than the second isoform (bPgA-1b), and 159.75 Da lower than the third one (bPgA-1c). In fraction 2 (bPgA-2), the first isoform has a MM of 40,104.70 Da (bPgA-2a) and it weighs 38.23 Da lower than the second isoform (bPgA-2b), and 76.69 Da lower than the third one (bPgA-2c). The calculation of phosphate content gave a range of 0.98-3.90 (Table 2).

**Protease activity:** The proteins in peaks 1, 2 and 3 of the HT column gave a similar protease activity in the method. The potential pepsin activity was higher when compared with that of human and pig pepsinogen (Table 3). Bovine chymosin B showed the lowest value in the same method.

## DISCUSSION

Pepsinogen is a zymogen with a great interest not only in food digestion (by its proteolytic activity in acidic pH) but also because it is measurable in blood circulation as an indicative parameter of the integrity of the gastric mucosa. The objective of this work was to purify the bovine pepsinogen A by an efficient, simple and reproducible protocol in order to obtain great quantities of pure and not denatured proteins; needed for the immunization of rabbits intended to produce antisera necessary for immunoassays development (RIA and ELISA).

After 40-70% ammonium sulfate fractionation, the procedure of purification involved DEAE ceramic and Hydroxyapatite as chromatographic columns. Three fractions with high activity were separated from HT column and appeared to have a relative molecular mass around 45kDa. The SDS-PAGE migration showed single band for peak 1 and 2, but the third peak of the HT elution presented other minor bands with greater mobility. The sequence analysis showed the same N-terminal for all HT peaks, and Mass Spectrometry revealed that peak 1 and 2 presented 6 different masses due to different phosphate contents. As measured in the indirect enzymatic test, the enzyme proteolytic activity contained in peaks 1, 2 and 3 of the HT column was high, greater than those of commercial human and porcine pepsinogen. And, the difference between the 3 peaks was not significant suggesting that phosphate content does not influence the potential enzymatic activity. The different controls, SDS-PAGE, sequence analysis, mass spectrometry and proteolytic activity clearly indicate the purity and the absence of denaturation of our proteins, so they are suitable for immunization of laboratory animals.

Bovine pepsinogen was first purified by Chow and Kassell[19] by a procedure involving more than five chromatographic steps. These authors, starting with very large quantities of extracts, used DEAE-cellulose as last chromatographic step, after ammonium sulfate fractionation and batch absorptions on DEAE-cellulose. They did a very interesting biochemical work in term of pepsinogen characterization and to its N-terminal sequencing preparation. Unfortunately, they did not report on immunization of laboratory animals for the production of specific antisera. The present procedure was performed to produce pure and nondenatured pepsinogen for antisera, tracer and standard production. Together with the high enzymatic activity, SDS-PAGE,

Table 3: Comparison of proteolytic activity of the purified bovine pepsinogen A isoforms and related commercial molecules.

| Proteolytic activity | Porcine pepsinogen A* | Human pepsinogen A* | Bovine chymosin B* | Bovine pepsinogen A-1 | Bovine pepsinogen A-2 | Complex Fraction 3 A-3 |
|---|---|---|---|---|---|---|
| Utyr/mg | 4.27 | 4.10 | 0.19 | 5.15 | 7.45 | 6.81 |

*: Purchased from Sigma Aldrich

microsequencing and mass spectrometry clearly showed the absence of pepsin and the high degree of homogeneity of our preparation. Indeed, after only two chromatographic steps, pepsinogen was obtained in a homogeneous form. Gomes *et al.*[14] obtained single form of bovine pepsinogen preparation after a DEAE cellulose and a Mono Q columns. Their protocol gave a relatively higher recovery than that of Chow and Kassell[19], otherwise the quantity of the starting material was so low that the results in term of pure protein available were very low. Due to the sensibility of pepsinogen to denaturation and its digestion into pepsin at acidic pH, the purification can not use cation exchange chromatography. In the present study, DEAE ceramic and Hydroxyapatite columns were chosen. Hydroxyapatite was first used for protein purification by Hjerten *et al.*[29]. Meitner and Kassell[21] used for the first time hydroxyapatite for bovine pepsinogen purification, after Sephadex G-100 and DEAE cellulose columns. These authors separated four peaks which had approximately the same potential specific activity and there were no differences in amino acid composition among them. Hydroxyapatite is characterized by an insoluble hydroxylated calcium phosphate [$Ca10(PO_4)6(OH)_2$] which forms both matrix and ligand. The functional groups consist of pairs of positively charged calcium ions referred to as C-sites and clusters of negatively charged phosphate groups, referred to as P-sites arranged in a crystalline structure. Hydroxyapatite provides thus a charge-based, mixed-mode functionality derived both from the composition and spatial arrangement of constituent atoms.

In agreement with the study of Meitner and Kassell[21], the present study showed similar proteolytic activities of the eluted isoforms which presented higher activity when compared with commercial human or pig pepsinogen A. The microsequencing gave the same N-terminal amino acids for each peak. The comparison of the obtained sequence with that reported by Harboe and Foltmann[27] or by Munoz *et al.*[28] showed total identity. Munoz *et al.*[28] determined the total nucleotide sequence of bovine pepsinogen A and it was over 372 amino acids. The percentage of identity between pepsinogen A isolated from human, pig and bovine abomasa (> 80%) showed a high degree of conservation of this zymogen.

Further more, slight difference was found between the relative molecular mass calculated after SDS-PAGE (45 kDa) and values reported by Chow and Kassell[19] (37.5 kDa) by sedimentation and ultracentrifugation methods. The molecular mass of bovine pepsinogen found in the present study is in agreement with the results of Gomes *et al.*[14] who reported a value 46 kDa after SDS-PAGE. Mass Spectrometry revealed 6 components in peak 1 and 2 differing in the phosphate content. Previous isolation of bovine pepsinogen[30] has yielded a product which consisted of four different types differing solely on the basis of the organic phosphate content of the enzyme, whereas Eckersall *et al.*[23] isolated three isoforms without mention about the phosphate content. It is likely that some of the present pepsinogen A isoforms, correspond to the forms previously reported[21]. In the present pepsinogen preparations, the organic phosphate content ranged between 1 and 4 mol/mol of protein, these values differ slightly from previous results (0.3-3 or 0.5-2.2 mol/mol of protein). Canine pepsinogen contains 0.89 mol/mol of protein[31]. In goats, 3 components of pepsinogen A have been isolated (A-1, A-2 and A-3) and the phosphorylation rate was from 1.42 to 2.00mol/mol of protein, and in ovine pepsinogen, the rate was from 1.5 to 2.36 mol/mol of protein[32]. In rabbits, four pepsinogens type A were isolated[33]. Human pepsinogen A also has 5 minor forms[34]. Phosphate which might be responsible for the multiplicity is shown to be present also in monkey pepsinogen[35].

Phosphorylation is a post-traductional modification which may have a role in protein transportation and localisation, protein function, interaction within proteins or cell signalling. Early studies found that protein kinases phosphorylated their target proteins at discrete sites, and only phosphorylated a limited number of available sites[36]. By sequence analysis by the software NetPhos 2.0 Server[37], bovine pepsinogen A revealed 24 available sites of phosphorylation (20 serine sites, 2 threonine sites and 2 tyrosine sites). According to the range of phosphate moles (1-4) determined in the present study, it is likely that only few sites were really phosphorylated in the pepsinogen molecule. Unless indicated, no attempt has been made to weigh information gained from proteins known to be phosphorylated *in vivo*. The molecular mass of bPgA determined on the basis of the cDNA was 39,876 Da[28], this value is in concordance with the MM of bPgA-1a (39,864.98 Da) suggesting that this form represents the nonphosphorylated pepsinogen A, thereafter the other forms correspond to different degrees of the phosphorylated molecule.

The present preparations of bovine pepsinogen kept their proteolytic activity towards bovine serum albumin as substrate, the zymogens revealed higher activity than porcine pepsinogen A and human pepsinogen A and, bovine chymosin presented very low activity in the same conditions. Previously, it has been reported that the bovine pepsins showed 60-70% of the activity of porcine pepsins with haemoglobin as substrate while the milk clothing action was similar, and chymosin showed 25% of porcine pepsins activity[30]. As the protease activity depends essentially on the nature of the substrate used and on the pH, the reported values are usually related to the assays conditions. Thus the high activity of the bovine pepsinogen A and the low activity of bovine chymosin found in the same conditions are in agreement with the earlier results.

Starting with bovine extracts, the protocol including ammonium sulphate fractionation, chromatography on DEAE and hydroxyapatite privileged the isolation of only pepsinogen A type. Otherwise, pepsinogen C type has been previously detected in bovine mucosal extract and it has been reported that, in terms of relative abundance of the zymogen, the ratio PgA to PgC was 30:1 whereas in human, the value was 3:1[23]. In their study, Antonini and Ribadeau-Dumas[22] isolated both bovine pepsinogen A and C after ammonium sulfate fractionation of 63% followed by four steps of chromatography. The current study found the highest proteolytic activity in the 40-70% ammonium sulfate fractionation and pepsinogen A type was isolated after only two chromatographic steps.

The main objectives were achieved, the bovine pepsinogen was obtained in homogeneous form, with a high proteolytic activity and in great quantity. In

## CONCLUSIONS

The procedure described in the present work, using complementary methodologies to purify and partial characterize bovine pepsinogen with a reduced number of steps, gives pure protein with high protease activity. The isoforms exhibit the same N-terminal and differ only in their degree of phosphorylation. These pure and active fractions are available for further investigations including the development of specific immunoassays as diagnostic tools. For this, the work is in progress and New Zealand White Rabbits are presently immunized for the production of specific antisera (the ethic authorization number: 297).

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