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Effects of rumen fluid pre-incubation on *in vitro* proteolytic activity of enzymatic extracts from rumen microorganisms

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ABSTRACT

Enzymatic extracts were prepared from rumen fluid (RF) either directly after collection or after incubation in enriched media and their proteolytic activities were measured and compared. The objective of RF *in vitro* cultivation was to augment enzymatic activity by using diverse incubation media containing different feed substrates to selectively induce microorganisms, with essentially unrestricted sources of energy and N. The effects on protein degradation in bovine serum albumin (BSA) and soybean meal, rapeseed meal, dehydrated alfalfa meal and perennial ryegrass were determined. Enzymatic extracts were characterized by means of protease zymograms. Two adult Holstein Friesian rumen fistulated cows were used as RF donors. Standard anaerobic incubation techniques in buffer-mineral media were used, and protein degradation was measured as residual true protein after enzymatic incubations. Results reveal an effect of RF pre-incubation on protein degradation ($P<0.0001$) measured in BSA and in feed substrates, and also on the hydrolysis rate ($P<0.001$), measured with native substrates only. The enzymatic extracts showed a high potential for proteolytic degradation, reaching 785 mg/g crude protein (CP) degraded in BSA after 12 h of incubation, and 830 mg/g CP degraded in soybean meal with 48 h of incubation, compared to only 654 mg/g and 414 mg/g in controls. The zymograms of these extracts revealed six zones of proteolytic activity, with the highest concentration of peptidases in the zone of high molecular weight (117 and 130 kDa). *In vitro* pre-incubation of rumen fluid in culture media enriched with energy and N sources allowed collection of enzymatic extracts with higher proteolytic activity than extracts obtained directly from fresh rumen fluid.

1. Introduction

Sustainable and efficient use of N resources in feeding ruminants requires accurate assessment of the dynamics of protein hydrolysis in the rumen. Diverse chemical and *in vivo* enzymatic methods have been developed in order to study these dynamics. Methods based on commercial enzymes (Pichard and Van Soest, 1977; Mahadevan et al., 1987; Assoumani et al., 1992) have used proteases of fungal origin (*i.e.*, *Streptomyces griseus*) and have reached high degradation levels and hydrolysis

Abbreviations: ADF, acid detergent fiber; ADIN, insoluble N in acid detergent fiber; BSA, bovine serum albumin; CP, crude protein; DAM, dehydrated alfalfa meal; DM, dry matter; Dmax, protein degradation (48 h); kd, hydrolysis rate; NDF, neutral detergent fiber; NDIN, insoluble N in neutral detergent fiber; PR, perennial ryegrass; RF, rumen fluid; RSM, rapeseed meal; SBM, soybean meal; TP, true protein.

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rates. However, some authors have stated that use of these non-ruminal proteases in *in vitro* conditions may have a limited predictive value, in what: due to possible substrate–enzyme specificity and uncertainty in how much these hydrolytic events are representative of the ruminal system (Roe et al., 1991; Luchini et al., 1996; Stern et al., 1997). The method of chemical fractioning of feed proteins (Sniffen et al., 1992; Licitra et al., 1996) adopted by the Cornell Net Carbohydrate Protein System (CNCPS, 1992) is a meritorious application of a routine and reliable procedure of chemical analysis in the laboratory. Nevertheless, it has been criticized for its methodological rigidity and limited capacity to simulate dynamics of degradation (Stern et al., 1997; Mustafa et al., 2001). A widely adopted method consists of *in sacco* intraruminal incubation (Mehrez and Ørskov, 1977; Ørskov, 1992), whose main merit is its similarity to the rumen. However, this method has been severely criticized due to errors and limitations resulting from: (a) bacterial contamination and microbial proteins that penetrate and remain in the fibrous residues of the bag, (b) generation of a modified environment inside the incubation bag due to a limited permeability to gases and solutions, (c) escape of undegraded proteins and particles from the bags into the ruminal medium and (d) the need to have fistulated animals and the limited number of samples that can be studied simultaneously (Mathis et al., 2001; Cone et al., 2004).

Some investigators have studied protein hydrolysis with enzymes extracted from RF (Mahadevan et al., 1987; Kohn and Allen, 1995a,b), obtaining remarkable levels of degradation of protein in early incubation times (*e.g.* 473 mg/g CP in soybean meal at 16 h of incubation). RF has the potential to supply enzymatic concentrates with proteolytic activity that could probably last more than 16 h of incubation (Kohn and Allen, 1995b). The reduced enzymatic activity obtained through ruminal enzymes compared to the *in sacco* intraruminal technique could be attributed to a low enzymatic concentration and/or to insufficient microbial diversity to generate enzymes needed for high protein hydrolysis. The persistence of the enzymatic activity of ruminal extracts in the incubation media have been suggested by Kohn and Allen (1995b) and Stern et al. (1997) as probable limitations to reach higher degradation values. In addition, they suggested that future investigations should characterize the raw extracts of these enzymes on different types of feed and evaluate other structural components or factors that could interfere with protein degradation. Although this methodology does not allow growth of substrate-specific rumen microbes, enzymes of ruminal origin would certainly have the potential to better simulate, under *in vitro* conditions, hydrolysis that occurs in the rumen. To this end, if a high enzymatic concentration could be maintained over a long period of time to ensure a sustained substrate-limited reaction, using an enzymatic extract with a broad action range, normal enzymatic activity could be obtained and allow enzymatic degradation of proteins complexed with other molecules or polymers such as structural or starch polysaccharides.

In order to augment the enzymatic concentration by promoting more microbial growth, an *in vitro* RF incubation without limitations in N or energetic sources is suggested. Accordingly, the objective of this investigation was to prepare ruminal enzyme extracts with high proteolytic activity, obtained through RF pre-incubation with different substrates to promote synthesis of a diverse group of enzymes.

2. Materials and methods

In the experiments described below, enzymatic extracts of microbial origin were prepared from RF. These extracts were processed directly from collected RF or after *in vitro* pre-incubation in media containing different substrates. In the first experiment, effect of pre-incubation on proteolytic activity of extracts was tested on bovine serum albumin (BSA) and the repeatability of this methodology was measured. In the second experiment, the proteolytic activity of the enzymatic extracts on different feed substrates was studied. In order to compare the patterns of molecular weight distribution, zymogram of proteases were compared in extracts from either precultured or directly collected RF.

2.1. Collection of ruminal fluid and in vitro pre-incubation

RF was obtained from two adult rumen fistulated cows fed at maintenance metabolizable energy level in two daily doses with a diet consisting of alfalfa hay (800 g/kg), coarsely ground maize grain (200 g/kg) and mineral salts (120 g/d), with CP making up 147 g/kg of the diet. Feeding and access to water were suspended 1 h before RF collection. Drinking water was available at all other times.

In the laboratory, the RF was handled under strict anaerobic conditions with constant CO_2 gasing. In order to reduce variability among RF collections, the ratio of liquid and solid fractions of the rumen contents was controlled by separating these fractions by filtration, then mixing them in equal weights of 1 kg each. Subsequently, the contents were homogenized and filtered according to the method of Goering and Van Soest (1970). The aim of this work focused on proteolysis, however RF pre-incubation was done with mixed substrates that provided plant proteins, plant cell walls, starch and sugars, in order to better simulate the complex microbial interactions that occur in the rumen as well as to build a broad enzymatic system. Chamberlain and Choung (1995) and Kornegay (1996) have shown in microbial consortia that proteolytic activity occurs simultaneously with amylolytic, hemicellulolytic or cellulolytic activities. For this purpose, each incubation was designed to encourage development of microbes with greater affinity to either protein, starch or cellulose by increasing the proportion of the target substrate, but smaller proportions of the other substrates were included to ensure diversity. The main pre-incubation substrates were ryegrass fiber (FDN), corn starch (Corn Starch Sigma EC 232-679-6) and a mix of soybean meal and alfalfa meal as protein sources (50/50) (Table 1). This *in vitro* pre-incubation was carried out with 100 ml of RF inoculum, 5 g of mixed substrates, 100 ml of macro and micromineral solution, 100 ml of sodium bicarbonate buffer (pH 6.8), 20 ml of