Proteolysis of Alcohol-Treated Soybean Meal Proteins by *Bacteroides ruminicola*, *Bacteroides amylophilus*, Pepsin, Trypsin, and in the Rumen of Steers

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Urbana 61801

ABSTRACT
Sodium dodecyl sulfate-gel electrophoresis and cation exchange chromatography were used to examine degradation of treated and untreated soybean meal protein fractions by *Bacteroides amylophilus* H18₁, *Bacteroides ruminicola* B₁₄, pepsin, trypsin, and intraruminally. Soybean meal treatments consisted of 30% vol/vol isopropanol, 40% propanol, or 50% ethanol at 22°C or 70% ethanol at 80°C. Water-soluble protein fractions were applied to a hydroxylapatite column and eluted with a discontinuous phosphate gradient of .03 to .27 and then .27 to 1.0 M. The four protein fractions with the highest absorbance at 276 nm were dialyzed against distilled water prior to being subjected to enzymatic hydrolysis. Soybean meal treated with 40% propanol had the greatest reduction in absorbance of all effluents at 275 nm, followed by soybean meal treated with 50% ethanol or 30% isopropanol. Comparison of electrophoretic patterns over time showed that *B. amylophilus* H18₁, degraded protein subunits more rapidly than *B. ruminicola* B₁₄. Protein subunits with the highest molecular weights were the most rapidly degraded by *B. amylophilus* H18₁, *B. ruminicola* B₁₄, pepsin, and trypsin. Hydroxylapatite chromatography of omasal fluid from steers supplemented with untreated soybean meal or soybean meal treated with 70% ethanol at 80°C indicated that no detectable soluble glycinin or conglycinin escaped ruminal degradation.

INTRODUCTION
Soybean meal (SBM) is a common protein source in ruminant diets and is rapidly degraded by ruminal microorganisms. Ruminal digestion of protein is less efficient than postruminal digestion (4) because some of the degraded protein N is lost from the rumen as ammonia. Ammonia is converted into urea in the liver and much of it is subsequently excreted in the urine. Various treatments have been examined for their ability to improve resistance of SBM protein to ruminal microbial proteases. Formaldehyde (19) and heat (9) treatments reduced degradation of SBM protein by ruminal microbes. However, excessive treatment resulted in depressed postruminal availability as measured by chick growth bioassay (20, 22). Soybean meal treatments involving alcohols, with and without addition of heat, depressed ruminal protein degradation (13, 24) but did not affect the nutritive value for chicks (12, 25). Development of a commercial process to extract soy flakes with aqueous isopropanol (1) generated interest in the effect of these solutions on susceptibility of soy protein to enzymatic degradation.

In vitro fermentation of different feed proteins with *Bacteroides amylophilus* indicated wide variation in the degradation rates of different soluble proteins, and the relationship between degradation rate of soluble and insoluble proteins is inconsistent (14). Reduced N disappearance in situ was observed when SBM was treated with aqueous alcohols (5, 12, 24); however, hydrolysis of alcohol-treated SBM protein by *Aspergillus sojae* occurred faster than that of untreated SBM (8).
PROTEOLYSIS OF ALCOHOL-TREATED SOYBEAN MEAL PROTEINS 2417

Effect of alcohol treatments on proteolysis of SBM proteins by ruminal microbial and mammalian proteases and on in vivo degradation of specific SBM proteins has not been reported before. The purpose of this study was to determine effects of various alcohol treatments on different SBM protein fractions and to measure proteolysis of these fractions by microbial proteases, pepsin, trypsin, and within the rumen.

MATERIALS AND METHODS

Soybean Meal Treatment

Hexane-extracted, dehulled SBM was treated with various aqueous alcohol solutions at two temperatures. Treatments with 30% vol/vol isopropanol, 40% vol/vol propanol, or 50% vol/vol ethanol were conducted at 22°C. These concentrations were chosen because earlier work (24) indicated that they resulted in the greatest depression in ruminal degradation. One hundred gram SBM were soaked in 250 ml of alcohol solution for 30 min. After soaking, SBM was filtered through Whatman No. 41 filter paper and air-dried. The heat processed treatment involved adding 22.7 kg SBM to 76.8 L of 70% vol/vol ethanol heated to 80°C. The mixture was stirred for 30 min, after which the liquid was drained and the SBM dried at 77°C for 12 h.

Soluble proteins were isolated from untreated SBM treated at 22°C with 30% isopropanol, 40% propanol, and 50% ethanol or from SBM treated with 70% ethanol at 80°C, or from omasal fluid from steers fed untreated SBM, 70% ethanol-treated SBM at 80°C, or urea-casein. Forty-gram SBM samples were suspended in 200 ml distilled water and agitated for 1 h in a 40°C water bath prior to centrifugation for 20 min. The supernatant was filtered through Whatman No. 41 filter paper and air-dried. The heat processed treatment involved adding 22.7 kg SBM to 76.8 L of 70% vol/vol ethanol heated to 80°C. The mixture was stirred for 30 min, after which the liquid was drained and the SBM dried at 77°C for 12 h.

Soluble proteins were isolated from untreated SBM treated at 22°C with 30% isopropanol, 40% propanol, or 50% ethanol from SBM treated with 70% ethanol at 80°C, or from omasal fluid from steers fed untreated SBM, SBM treated with 70% ethanol at 80°C, or urea-casein. Forty-gram SBM samples were suspended in 200 ml distilled water and agitated for 1 h in a 40°C water bath prior to centrifugation for 20 min. The supernatant was exhaustively dialyzed against .03 M potassium phosphate buffer containing .1% α-mercaptoethanol, followed by gel filtration over a Sephadex G-25 column (Pharmacia Fine Chemicals, Inc. Milwaukee, WI). An amount of extract containing 200 mg protein (10) was applied to the hydroxylapatite column. The column was eluted with a discontinuous phosphate gradient of .03 to .27 M then .27 to 1.0 M (6) using an Ultragrad (LKB-Produkter AB, S-16125 Bromma, Swed.) gradient mixer. Proteins eluting from the column were detected by monitoring column effluent at 276 nm with a Unicord S monitor (LKB-Produkter AB). The four largest fractions from untreated SBM and SBM treated with 30% isopropanol, 40% propanol, and 50% ethanol at 22°C were extensively dialyzed against distilled water, frozen, and stored. Soluble proteins in omasal fluid from steers fed untreated SBM, 70% ethanol-treated SBM at 80°C, or urea-casein were prepared by compositing samples by treatment, followed by centrifugation at 35,000 × g, with the supernatant dialyzed against .03 M phosphate buffer containing .1% α-mercaptoethanol. Proteins were separated as above by applying 24 mg protein to the hydroxylapatite column. Omasal fluid was collected as described previously (13).

Amino Acid Analysis

Fractions isolated from untreated SBM were hydrolyzed with 6N HCl for 22 h at 110°C prior to analysis for amino acid composition with a Beckman 119CL amino acid analyzer (Beckman Instruments, Inc., Palo Alto, CA). Release of α-amino groups was determined as described by (17). Digestion rates of various protein fractions were expressed as micrograms glycine equivalents released per milligram enzyme protein-1·minute-1.

In Vitro Bacterial Digestion

The bacteria, Bacteroides amylophilus strain H181 and Bacteroides ruminocola strain B14, were grown and maintained on media described by Schaefer et al. (21). Eighteen hours after transfer to fresh media, bacteria were centrifuged at 8000 × g. The pellet containing the bacteria was suspended in 1 ml .05 M phosphate buffer (pH 7.6). Bacterial cells were disrupted using a French press cell (20,000 psi) and suspended in 25 ml .05 M phosphate buffer (pH 7.6). Three milliliters of water-soluble protein extract were incubated with 1 ml bacterial suspension. After 1, 3.5, and 6 h of digestion, 1-ml samples were immediately analyzed for free α-amino groups. Rates of protein hydrolysis for the protein fractions were determined by linear regression.

In Vitro Trypsin and Pepsin Digestion

Three milliliters of water-soluble protein extract from untreated SBM and 30% iso-
propanol, 40% propanol, and 50% ethanol-treated SBM were incubated with .10 mg trypsin in 1 ml .05 M phosphate buffer (pH 7.6) at 25°C. After 1, 4, and 7 min, 1-ml samples were taken and digestion was stopped by adding .1 ml of 1 N HCl. Prior to analysis for free α-amino groups, .1 ml 1 N NaOH was added to the tubes. Rates of protein hydrolysis were determined by linear regression.

To evaluate susceptibility to pepsin, 3 ml of water-soluble protein extract fractions were incubated with .4 mg pepsin in 1 ml 1 N HCl at 30°C. After 1, 9, and 17 min, 1-ml samples were taken, neutralized with .2 N NaCl and analyzed for free α-amino groups. Rates of protein hydrolysis were determined by linear regression.

Electrophoresis

Composition of protein fractions isolated from untreated SBM extract was examined using SDS PAGE (26). Also, effects of hydrolysis by various proteases on electrophoretic patterns of SBM protein over time were studied. Hydrolysis conditions were similar to those described previously. The molecular weight of subunits was determined with a standard protein solution consisting of lysozyme (Mr 14,300), trypsinogen (Mr 24,000), ovalbumin (Mr 45,000), bovine plasma albumin (Mr 66,000), and phosphorylase a (Mr 92,500). Pepsin and trypsin digestions were stopped after 0, 1, 2, 4, 10, 30, and 60 min. Bacterial digestions were stopped after 0, 4, 8, 12, and 24 h of digestion.

Ruminal Protein Degradation

Three mature Hereford steers (average 508 kg) and three mature Simmental steers (average 594 kg) fitted with ruminal cannulae were used to measure soluble SBM protein outflow from the rumen. Treatments consisted of three N supplements: untreated SBM, SBM treated with 70% ethanol at 80°C, and a urea-casein-corn mix. Nitrogen supplements were added to a basal diet (Table 1) to achieve a 14.4% CP final diet, with approximately 70% of the dietary CP provided by N supplements. Steers were fed daily at 0800 and 2000 h and were tethered in solid floor stalls in a temperature-controlled (18.3°C) room under continuous lighting.

Each experimental period was 14 d in length. Days 1 through 10 were for diet adaptation with d 11 through 14 used for collection of omasal fluid samples. Cobalt EDTA (23) was continuously infused into the rumen to serve as omasal fluid flow marker. Approximately 75 ml of omasal fluid were collected five times daily; collection times were randomized so that omasal samples were collected every 70 min of a 24-h period. Omasal samples were collected by inserting a polyethylene tube (.95 cm i. d., 1.59 cm o. d.) into the omasum through the reticulo-omasal orifice. A plastic strainer was attached to the omasal end of the tube and held approximately 12 cm into the omasum by 2 × 10 cm rubber flanges positioned on each side of the reticulo-omasal orifice. Fluid samples were collected by attaching the opposite end of the tube to a filter flask and applying negative pressure with a vacuum pump. Omasal samples were composited, centrifuged at 14,000 × g, and frozen at −20°C prior to protein isolation.

Statistical Analysis

Data on rate of digestion were analyzed by analysis of variance. When significant differences were observed, treatment means were compared by the F test-protected, least significant difference method (3).

RESULTS

Elution patterns resulting from separation of water-extractable proteins on the hydroxylapatite column from untreated SBM or from SBM treated with 30% isopropanol, 40% propanol, and 50% ethanol are presented in Figures 1, 2, and 3. Four major and two minor peaks were observed. The first fraction eluted between .03 and .05 M phosphate, the second between .18 and .23 M phosphate, the third with .27 M phosphate, and the fourth between .4 and .7 M phosphate. Two minor peaks were observed between .08 and .12 M and .23 and .25 M phosphate.

The relative absorbance at 276 nm of soluble protein fractions from SBM treated with 30% isopropanol is superimposed on the pattern found for untreated SBM in Figure 1. The isopropanol-treated sample contained more proteins that eluted at .03 M and between .18 and .23 M phosphate and less of the proteins...
TABLE 1. Ingredient and chemical composition of basal diet (dry basis).

<table>
<thead>
<tr>
<th>Item</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient composition</td>
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</tr>
<tr>
<td>Corncobs, ground</td>
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</tr>
<tr>
<td>Cornstarch grits</td>
<td>30.14</td>
</tr>
<tr>
<td>Prairie hay, ground</td>
<td>12.5</td>
</tr>
<tr>
<td>Dextrose</td>
<td>11.0</td>
</tr>
<tr>
<td>Casein</td>
<td>.5</td>
</tr>
<tr>
<td>Urea</td>
<td>.4</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>.2</td>
</tr>
<tr>
<td>Limestone</td>
<td>.16</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>.1</td>
</tr>
<tr>
<td>Chemical composition</td>
<td></td>
</tr>
<tr>
<td>Dry matter</td>
<td>79.7</td>
</tr>
<tr>
<td>Crude protein</td>
<td>4.31</td>
</tr>
<tr>
<td>Acid detergent fiber</td>
<td>26.4</td>
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</table>

1 Contains 3,300,000 IU vitamin A, 330,000 IU vitamin D, and 22,000 IU vitamin E/kg.

The same observations were made for the samples from SBM treated with 40% propanol (Figure 2) and 50% ethanol (Figure 3). Reduction in peak 3 and 4 proteins was largest for the 40% propanol treatment. Fifty percent ethanol treatment resulted in a reduction in size of these peaks intermediate between isopropanol and propanol.

Four fractions of the water-extractable proteins from untreated SBM were obtained as shown in Figure 1. The amino acid composition of these fractions is presented in Table 2. Fraction 1 contained high concentrations of threonine, alanine, and glycine and low concentrations of leucine relative to the crude SBM protein fraction applied to the column. Fraction 2 contained relatively lower concentrations of serine, leucine, tyrosine, and phenylalanine. Fraction 3 was the only one in which mea-

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Figure 1. Absorbance (276 nm) pattern of soybean meal proteins (—) applied to an hydroxylapatite column and eluted with a discontinuous phosphate buffer (-----) in comparison to the elution pattern for soybean meal treated with 30% isopropanol (-----).
Figure 2. Absorbance (276 nm) pattern of soybean meal proteins (-----) applied to an hydroxylapatite column and eluted with a discontinuous phosphate buffer (-----) in comparison to the elution pattern for soybean meal treated with 40% (vol/vol) propanol (--.--.--).
TABLE 2. Amino acid composition of protein fractions isolated from untreated soybean meal (nmol/mg total protein).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Crude fraction</th>
<th>Fraction 1</th>
<th>Fraction 2</th>
<th>Fraction 3</th>
<th>Fraction 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1526</td>
<td>1763</td>
<td>1178</td>
<td>1334</td>
<td>1463</td>
</tr>
<tr>
<td>Threonine</td>
<td>422</td>
<td>910</td>
<td>247</td>
<td>389</td>
<td>284</td>
</tr>
<tr>
<td>Serine</td>
<td>729</td>
<td>1073</td>
<td>314</td>
<td>675</td>
<td>820</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>2743</td>
<td>1486</td>
<td>2457</td>
<td>2045</td>
<td>8683</td>
</tr>
<tr>
<td>Proline</td>
<td>695</td>
<td>538</td>
<td>707</td>
<td>678</td>
<td>131</td>
</tr>
<tr>
<td>Glycine</td>
<td>823</td>
<td>2175</td>
<td>608</td>
<td>811</td>
<td>814</td>
</tr>
<tr>
<td>Alanine</td>
<td>644</td>
<td>1480</td>
<td>302</td>
<td>566</td>
<td>334</td>
</tr>
<tr>
<td>Valine</td>
<td>296</td>
<td>325</td>
<td>123</td>
<td>344</td>
<td>301</td>
</tr>
<tr>
<td>Methionine</td>
<td>83</td>
<td>ND</td>
<td>ND</td>
<td>46</td>
<td>ND</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>269</td>
<td>247</td>
<td>112</td>
<td>238</td>
<td>300</td>
</tr>
<tr>
<td>Leucine</td>
<td>748</td>
<td>242</td>
<td>154</td>
<td>692</td>
<td>816</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>246</td>
<td>288</td>
<td>37</td>
<td>205</td>
<td>203</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>391</td>
<td>256</td>
<td>94</td>
<td>343</td>
<td>497</td>
</tr>
<tr>
<td>Histidine</td>
<td>161</td>
<td>193</td>
<td>183</td>
<td>148</td>
<td>119</td>
</tr>
<tr>
<td>Lysine</td>
<td>567</td>
<td>296</td>
<td>509</td>
<td>408</td>
<td>538</td>
</tr>
<tr>
<td>Arginine</td>
<td>597</td>
<td>433</td>
<td>491</td>
<td>531</td>
<td>600</td>
</tr>
</tbody>
</table>

1 Water-soluble proteins extracted from soybean meal with water at 40°C.
2 ND = Not detectable.

Figure 4. The SDS-PAGE patterns of the various separated protein fractions: 1) Fraction 1, 2) Fraction 2, 3) Fraction 3, and 4) Fraction 4.

Figure 5. The SDS-PAGE patterns of soybean meal protein after 0, 4, 8, 12, and 24 h digestion with Bacteroides amylobilus H18 extract at 25°C.
Figure 6. The SDS-PAGE patterns of soybean meal protein after 0, 4, 8, 12, and 24 h digestion with Bacteroides ruminicola B4 extract at 25°C.

Figure 7. The SDS-PAGE patterns of soybean meal protein after 0, 1, 2, 4, 10, 30, and 60 min trypsin digestion at 25°C.

Figure 8. The SDS-PAGE patterns of soybean meal protein after 0, 1, 2, 4, 10, 30, and 60 min pepsin digestion at 25°C.

Sizable amounts of methionine were detected. Fraction 4 was extremely rich in glutamic acid but low in proline.

Band patterns, as determined by SDS-PAGE, of the pooled treatments are shown in Figure 4. Fraction 1 did not contain determinable proteins. Electrophoresis of fraction 2 resulted in a wide, vague band with no distinguishable protein bands. This was probably caused by the presence of one or more glycoproteins. The SDS-PAGE pattern of fraction 3 showed several sharp bands. These bands all had molecular weights less than 35,000. Fraction 4 contained four major protein subunits and various minor ones with molecular weights ranging from 35,000 to 75,000 daltons.

Exposure of water-extractable SBM proteins to B. amylophilus H18, resulted in a gradual degradation of protein fraction subunits represented in bands A, B and C after 24 h digestion (Figure 5). A slight accumulation of lower molecular weight proteins could also be noted (band E).

Digestion of water-extractable proteins by B. ruminicola B4 resulted in SDS-PAGE patterns shown in Figure 6. Disappearance of bands was slower than noted for B. amylophilus. None of the bands had completely disappeared after 24 h of digestion. However, a reduction of bands representing the highest molecular weight subunits (band A) was again noted.

Hydrolysis with trypsin resulted in rapid digestion of the higher molecular weight protein subunits (Figure 7). Intensity of bands A and B, representing proteins with molecular weights of 78,000 and 72,000 daltons, respectively, began to diminish 1 min after adding the trypsin. After 10 min, band C began to lose intensity. None of the other protein bands appeared to be affected by this enzyme during these short incubation times.

The SDS-PAGE patterns during pepsin digestion are shown in Figure 8. In this assay, none of the bands completely disappeared. Bands representing the two highest molecular weight subunits (bands A and B) had lost intensity after 60 min of pepsin digestion.

No significant interactions between protein fraction rate of hydrolysis and alcohol treatment were detected. Changes in affinities of
TABLE 3. Effects of various alcohol treatments on rate of hydrolysis of soybean meal protein by various proteases.

<table>
<thead>
<tr>
<th>Alcohol treatment</th>
<th>Protease</th>
<th>Untreated</th>
<th>30% (vol/vol) isopropanol</th>
<th>40% (vol/vol) propanol</th>
<th>50% (vol/vol) ethanol</th>
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<tbody>
<tr>
<td></td>
<td>B. ruminicola</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. ruminicola</td>
<td>0.0081</td>
<td>0.0065</td>
<td>0.0063</td>
<td>0.0058</td>
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<tr>
<td></td>
<td>B. ruminicola</td>
<td>(.0018)²</td>
<td>(.0018)</td>
<td>(.0023)</td>
<td>(.0018)</td>
</tr>
<tr>
<td></td>
<td>B. amylophillus</td>
<td>0.0182</td>
<td>0.0213</td>
<td>0.0245</td>
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</tr>
<tr>
<td></td>
<td>B. amylophillus</td>
<td>(.0016)</td>
<td>(.0020)</td>
<td>(.0020)</td>
<td>(.0020)</td>
</tr>
<tr>
<td></td>
<td>B. amylophillus</td>
<td>1.98</td>
<td>2.30</td>
<td>2.31</td>
<td>2.58</td>
</tr>
<tr>
<td></td>
<td>B. amylophillus</td>
<td>(.39)</td>
<td>(.48)</td>
<td>(.62)</td>
<td>(.57)</td>
</tr>
<tr>
<td></td>
<td>B. amylophillus</td>
<td>9.76</td>
<td>16.49</td>
<td>11.86</td>
<td>15.13</td>
</tr>
<tr>
<td></td>
<td>B. amylophillus</td>
<td>(3.10)</td>
<td>(2.92)</td>
<td>(4.82)</td>
<td>(4.25)</td>
</tr>
<tr>
<td></td>
<td>Pepsin³</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pepsin³</td>
<td>1.98</td>
<td>2.30</td>
<td>2.31</td>
<td>2.58</td>
</tr>
<tr>
<td></td>
<td>Pepsin³</td>
<td>(.39)</td>
<td>(.48)</td>
<td>(.62)</td>
<td>(.57)</td>
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<tr>
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<td>Traspin³</td>
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<td>16.49</td>
<td>11.86</td>
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<td></td>
<td>Traspin³</td>
<td>(3.10)</td>
<td>(2.92)</td>
<td>(4.82)</td>
<td>(4.25)</td>
</tr>
</tbody>
</table>

¹ Data are expressed as grams glycine equivalents released -1 .milligrams enzyme protein -1 .minute -1 .
² Standard error of the mean values.
³ Obtained from Sigma Co., St. Louis, MO.

proteases for specific protein fractions were similar among treatments. Table 3 presents results of various alcohol treatments on rate of hydrolysis of SBM protein by proteases. Alcohol treatments did not affect SBM protein availability as measured by susceptibility of SBM protein to degradation by proteases. However, proteolysis by B. amylophillus extract was faster than that by B. ruminicola extract. Rates of hydrolysis by the various enzymes were not statistically comparable because they were determined under different conditions.

In Table 4, rates of proteolysis of various protein fractions are presented. Fractions 2 and 3 had more (P<.05) α-amino groups released per unit of time by B. ruminicola than did the crude fraction. Fraction 3 was hydrolyzed faster (P<.05) by B. amylophillus than either fraction 1 or the crude fraction. Fractions 3 and 4 were hydrolyzed faster (P<.01) by pepsin.

TABLE 4. Rate of hydrolysis of soybean meal protein fractions by various proteases.

<table>
<thead>
<tr>
<th>Protease</th>
<th>Crude fraction</th>
<th>Fraction</th>
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<td>B. ruminicola</td>
<td>.0016a</td>
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<tr>
<td>B. ruminicola</td>
<td>(.0020)³</td>
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<td>B. amylophillus</td>
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<tr>
<td>Traspin³</td>
<td>(4.05)</td>
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</tbody>
</table>

a,b,cValues in the same row with different superscripts differ (P<.05).
¹ Data are expressed as grams glycine equivalents released -1 .milligrams enzyme protein -1 .minute -1 .
² Variation in assay results made values obtained unreliable and were therefore considered as nondeterminable (ND).
³ Standard error of the means.

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Figure 9. Absorbance (276 nm) pattern of untreated soybean meal soluble proteins (---) and soluble protein from soybean meal treated with 70% ethanol at 80°C (- - - - - -) applied to a hydroxylapatite column and eluted with a discontinuous phosphate gradient (-- -- --).

than was fraction 2 or the crude fraction. The crude fraction applied to the hydroxylapatite column and fraction 3 were hydrolyzed more slowly (P<.05) by trypsin than were fractions 2 and 4.

Elution pattern of soluble proteins from untreated SBM and SBM treated with 70% ethanol at 80°C was similar to that of the other SBMs (Figure 9). Elution patterns of soluble proteins collected at the omasum of steers fed untreated SBM, SBM treated with 70% ethanol at 80°C, or urea-casein were all similar (Figure 10). All three N supplements resulted in increases in peak 1 with no detectable traces of peaks 3 or 4. Separation of samples applied to the hydroxylapatite column by SDS-PAGE produced no determinable proteins (data not shown).

DISCUSSION

Soluble proteins extracted from untreated SBM had an absorbance pattern at 276 nm similar to that reported previously (6, 28). The fraction eluting at .03 M phosphate (peak 1) is composed predominantly of trypsin inhibitors and metabolic proteins (18). Peak 2, eluting at .03 to .27 M phosphate, is possibly composed of a mixture of storage and metabolic proteins (18). The fraction eluting from hydroxylapatite at .27 M phosphate (peak 3) was identified as the major soybean storage protein, glycinin (6). Fisher et al. (6) identified the fraction eluting between .4 and .7 M phosphate (peak 4) as conglycinin but did not report a major peak eluting from the column when the phosphate buffer concentration was between .18 and .23 M as was observed in our work.

The greatest denaturation of glycinin and conglycinin was observed for the 40% propanol treatment (Figure 2). Previous reports (7) showed that treatment with 40% propanol resulted in more protein denaturation than did treatment with 50% ethanol. The 40% propanol treatment was more effective in denaturing conglycinin than was propanol at any other concentration (26).

Comparison of SDS-PAGE patterns of SBM proteins following exposure to bacterial protease showed that hydrolysis of protein sub-units occurred faster when *B. amylophilus* H18
Figure 10. Absorbance (276 nm) pattern of omasal fluid from steers fed untreated soybean meal (-----), soybean meal treated with 70% ethanol at 80°C (- - - - -), or urea-casein-corn (----- -----) applied to a hydroxylapatite column and eluted with a discontinuous phosphate gradient (- - -).

extract was used than when *B. ruminicola* Bt 4 was used. Distinct disappearance of these proteins was noticeable after 4 h with *B. amylophilus* (Figure 5), whereas bands were still visible after 24 h when SBM protein was incubated with *B. ruminicola* extract.

Enzymatic hydrolysis of SBM protein, as examined by SDS-PAGE, showed that the highest molecular weight subunits (71,000 and 78,000 daltons) were always the most rapidly digested. Comparison of pattern of pepsin and trypsin digestion over time (Figures 7 and 8) showed that proteolysis by trypsin was faster than that by pepsin and was probably due to the difference in specific activity of the enzymes used. At 25°C, glycycin was hydrolyzed more rapidly by pepsin than by trypsin (11). We were not able to confirm this finding. However, in our experiment, as well as in the previous work (11), protein subunits with the highest molecular weights were the most rapidly digested.

Rate of in vitro α-amino group release and the change in SDS-PAGE patterns show conformity. In both assays, the most rapid rate of digestion was found for trypsin. Comparison of the two bacterial proteases showed that *B. amylophilus* extract had a greater specific activity than did *B. ruminicola* extract. Based on changes in SDS-PAGE patterns, we expected that fraction 4 (conglycinin) would be the most rapidly digested of all fractions tested. This deviation from expected results is possibly due to variability in the in vitro protease assays. Therefore, we think the results of the SDS-PAGE assays are the most reliable of the two data sets.

Rate of in vitro ammonia release in ruminal fluid has been directly related to the composition of SBM proteins (26). It has been
observed that fractions relatively rich in con-
glycinin and acidic subunits of glycinin released
the greatest amounts of ammonia. van der Aar
et al. (26) hypothesized that conglycinin was
more susceptible to degradation by ruminal
bacterial proteases than the other proteins. The
preferential hydrolysis of conglycinin by B.
amylopbilus (Figure 5) and to a lesser extent by
B. ruminicola (Figure 6) support this hypo-
thesis.

Lack of differences in hydroxylapatite
elution patterns of omasal soluble proteins
from steers fed untreated SBM, SBM treated
with 70% ethanol at 80°C, and urea-casein-corn
indicate that the protein material is of pre-
dominantly bacterial origin. It was hypothe-
sized that some of the soluble glycinin and
conglycinin, once denatured by alcohol treat-
ment, would escape ruminal degradation and
flow out of the rumen. Because only 5 to 10% of
protein from alcohol-treated SBM was
soluble (13, 27), diets were formulated to allow
N supplements to provide 70% of dietary
CP. We thought this would provide measurable
soluble proteins if alcohol treatment resulted in
soluble proteins resistant to ruminal degrada-
tion. Because over 80% of SBM N is water-
extractable at pH 6.5 (28), the potential exists
for significant amounts of soybean proteins to
be solubilized in the rumen. Mahaevan et al.
(14) reported that in vitro degradation rates of
soluble and insoluble SBM proteins were
identical. Because previous work showed
increased ruminal outflow of SBM N when SBM
was treated with ethanol and heat (13, 27), it
was hypothesized that soluble proteins from
SBM treated with 70% ethanol at 80°C may
also escape the rumen. If the proteins escaping
the rumen could be captured and identified,
this information could be useful in determining
what qualities would be advantageous for
the ruminal escape of proteins. The soluble
proteins from steers fed urea-casein-corn are
thought to be of bacterial origin, since ruminal
half-life of casein is less than 22 min (15) and
very small amounts of protein from the corn
would be soluble.

Conglycinin is both rapidly hydrolyzed by
proteases and easily denatured by aqueous
alcohol solutions (16). The molecular three-
dimensional structure is responsible for these
properties. Conglycinin is glycosylated (2) and
thus has hydrophilic characteristics. These
hydrophilic properties cause the molecule to be
less compact when solubilized in water (7). This
loose structure promotes denaturation by
aqueous alcohol solutions and enzymatic
hydrolysis. Although changes due to alcohol
treatment reduce degradation of insoluble
proteins, they do not seem to provide sig-
nificant structural changes to prevent degrada-
tion of the soluble fraction in vivo. It appears
that increases in ruminal escape of alcohol-
treated SBM may be due to the proteins re-
duced solubility, since proteins that are rapidly
hydrolyzed by ruminal proteases are also most
susceptible to alcohol denaturation.

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