Extracellular Enzymes of *Micropolyspora faeni* Found in Moldy Hay

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Fully active enzymes with chymotryptic activity were demonstrated in moldy hay samples where *Micropolyspora faeni* was found as the predominant saprophyte by using polyacrylamide gel electrophoresis. Great quantitative differences in enzymatic activity were found among moldy hay samples.

The fact is well established that the inhalation of organic substances may produce respiratory distress in man and animal. The allergic nature of such clinical manifestations is generally accepted and can be explained on the basis of our present knowledge of immunology.

A classical example is the extrinsic allergic alveolitis called farmer's lung, which is observed in man and cattle exposed to moldy hay (14, 15, 18, 22). It is generally accepted that the causative allergens are found in moldy hay and are mostly derived from *Micropolyspora faeni*, a thermophilic actinomycete.

The detection of precipitins in patients against these allergens gave some evidence that the alveolitis could be attributed principally to the type III Arthus reaction, with the possible involvement of other immunological reactions such as the cell-mediated response which we demonstrated in cattle (S. Lazary, J. Nicolet, E. Rivera, and M. Wanner, Res. Vet. Sci., in press.)

Since many exposed subjects may harbor such precipitins without apparent clinical manifestations, the actual significance of the precipitins in the allergic manifestation is not quite clear. Thus, the presence of precipitins against *M. faeni* has to be interpreted principally as an expression of exposure to moldy hay. However, this immunological response provides a lead towards studying the antigens involved and thus possibly affords a better understanding of the pathogenesis of this respiratory illness.

Toward that end, the observations of Walbaum et al. (26) and Biguet et al. (1) are very pertinent. They found that farmer's lung patients usually produce precipitins against metabolic products of *M. faeni*. Some of these are enzymes such as carboxyesterases, lipase, and phosphatase. However, the precipitins are chiefly against enzymes with chymotrypsin-like activity. We were able to demonstrate a similar reaction in cattle (16). This immunological reaction against metabolic products of *M. faeni* was later reaffirmed by Fletcher et al. (6) and then by Edwards (5).

These findings raise the question of the contaminating source of such metabolic products of *M. faeni*, as there is no evidence that the inhaled spores or mycelia of this organism do invade the lung tissue or that they are metabolically active in the lung alveoli.

It is a well-known fact that the natural growth of *M. faeni* on hay as a substrate not only leads to an abundant production of mycelia and spores but also constitutes an attack on the hay substrate leading to the degradation of its constituents (8, 10). The result of this phenomenon is moldy hay with its characteristic degraded appearance and the presence of a white dust. The latter consists principally of organic products from the degraded hay, the metabolic products of the saprophyte *M. faeni*, and the mycelia and spores of this organism.

The purpose of this study was to detect the contaminating source of such metabolic products of *M. faeni*, namely, the extracellular enzymes with chymotrypsic activity, found in moldy hay extracts which will stimulate an immunological response in exposed subjects.

**MATERIALS AND METHODS**

**Hay samples.** We selected six samples of moldy hay from six different farms in four endemic areas where the occurrence of extrinsic allergic alveolitis in man (samples 3, 4, and 6) or in cattle (samples 1, 2, and 5) has been observed. By way of comparison, we examined two samples of good hay, one with the macroscopic appearance of good quality from a farm in an endemic area but without any history of extrinsic allergic alveolitis (sample 7), and the other from a nonendemic area (sample 8).

The microbiological examinations of the moldy hay samples were performed by R. Corbaz of the Swiss Federal Station for Agricultural Research, CH-1260...
Nyon, who found, in addition to the classical microflora (4, 11), a predominant occurrence of *M. faeni*. He was not able to detect the presence of *M. faeni* in the good hay samples.

**Hay extracts.** Aqueous extraction of all the hay samples was carried out according to the method of Pepys et al. (19), using Coca's fluid; the extracts were lyophilized. For experiments we used a 50-μg/ml concentration in physiological saline solution.

**Polyacrylamide gel disc electrophoresis.** Analytical disc electrophoresis was carried out by the method of Biozzi et al. (2, 3) by using a Pleuger acrylporph apparatus. Forty microliters of the extracts was subjected to electrophoresis with a current of 5 mA per tube for 30 min. After electrophoresis, the gel was carefully removed from the tube and stained for chymotrypsin activity. *N*-acetyl-DL-phenylalanine-beta-naphthyl ester (Schwarz/Mann, Kitts, New York) served as substrate and Fast blue salt B (Merck, Darmstadt, Germany) was used as the stain, according to the method of Uriel (25).

**Immunoelectrophoresis.** The immunoelectrophoresis test was performed by the method described by Walbaum et al. (26). Hay extracts were used as solutions of 50 μg of lyophilized extract in 1 ml of saline. Rabbit immune serum was produced by the method of Walbaum et al. (26) against a glycin extract (12) of *M. faeni* strain 9535 grown on V8 agar for 6 days at 40 C (12). The washed and dried plates were stained for chymotrypsin activity.

**Disc immunoelectrophoresis.** Disc electrophoresis was performed as described under polyacrylamide gel electrophoresis according to the method described by Maurer (13). *M. faeni* antisera was the same as used for the immunoelectrophoresis test.

**Enzyme and protein assays.** Determination of protein in the hay extracts was based on the Hartree (9) modification of the Lowry method. Bovine serum albumin (Fluka, Buchs, Switzerland) was used to plot a standard curve. For the assays, 2 mg of saline per ml of the lyophilized extracts was used. Due to the presence of pigment in the extracts, a blank was prepared for each sample. The extracts for the blank were heated in a water bath at 100 C for 60 min and then precipitated with 5% trichloroacetic acid. Each supernatant was dialyzed against saline and used as the blank. For the quantitative estimation of chymotrypsin activity, 2 mg of lyophilized hay extract was dissolved in 1 ml of saline. The activity was assayed with *N*-benzoyl-DL-phenylalanine-beta-naphthyl ester (Sigma Chemical Co., St. Louis, Mo.) as substrate, by a modification of the method of Ravin et al. (23). To 0.5 ml of the test solution, 2.5 ml of the substrate (0.1 mg/ml in 0.05 M sodium diethyl barbiturate buffer, pH 7.8) was added. The reaction mixture was incubated at 4 C. After 1 h, 0.5 ml of a freshly prepared solution of Fast blue salt B (4 mg of distilled water per ml) was added. After 3 min, 0.5 ml of 80% trichloroacetic acid was added. The reddish pigment was extracted by shaking the reaction mixture in 5 ml of ethyl acetate. The color density was measured against a reaction mixture in which the extract had been substituted by saline on a Hitachi ultraviolet Vis spectrophotometer at 550 nm. From a calibration curve of pure beta-naphthol, the color density was converted to micrograms of beta-naphthol. The unit of activity was defined as micrograms of beta-naphthol released in 1 h at 4 C. A blank in which the heated extract had been precipitated with trichloroacetic acid was used as a specific extract control for each sample.

**Metabolic substances of *M. faeni* grown on synthetic medium.** Strain 9535 of *M. faeni* was grown on V8 agar according to the method of Walbaum et al. (26, 27) at 40 C for 6 days. We modified the method by placing a sterile cellophane sheet on the surface of the agar. The culture was then inoculated on the cellophane sheet and incubated. To obtain the metabolic products of the culture, we discarded the cellophane sheet with the culture and froze the agar overnight at −20 C. We then thawed this agar over a Buchner filter under gentle pressure. The fluid containing the metabolic products of the culture was dialyzed against running tap water for 48 h and then lyophilized. We used this extract at a concentration of 30 mg of saline per ml.

**Experimental hay culture.** This was accomplished by inoculating *M. faeni* culture (strain 9535) on chopped sterilized hay (sample 8) by using the method described by Pepys et al. (21). We incubated the culture for 6 days at 40 C. These were then extracted and lyophilized as described for the other hay samples.

**RESULTS**

**Chymotrypsin activity in polyacrylamide gel.** Figure 1 shows that moldy samples (no. 1, 2, and 4) are rich in different fractions with enzymatic activity on the chymotrypsin substrate *N*-acetyl-DL-phenylalanine-beta-naphthyl ester. Sample 4 presents the strongest reaction. Sample 1 is comparable but with a better-stained, slow-migrating band. Other moldy hay samples which are not included in Fig. 1 (no. 3, 5, and 6) possess only two bands with strong enzymatic activity and, like sample 2, are towards the anode. Good hay sample 7 shows one band with chymotrypsic activity but only weakly stained; the other good hay sample (extract 8) does not show this band. For comparison we present the patterns of chymotrypsin activity in the metabolic products of *M. faeni* on a synthetic medium and in experimental hay culture. These patterns show similarities to those of the moldy samples.

**Quantitative estimation of protein content and enzymatic activity.** The results of studies of moldy and good hay samples, metabolic products of *M. faeni* on synthetic medium and on hay culture are summarized in Table 1.

**Immunological identification of the chymotrypsin-like enzymes.** Disc immunoelectrophoresis. The immunoprecipitation pattern in agar gel for sample 4 between the electrophoretically separated extracts in polyacrylam-
EXTRACELLULAR ENZYMES OF M. FAENI

***Fig. 1.*** Enzymes with chymotrypsin-like activity revealed in polyacrylamide gel disc electrophoresis (substrate: N-acetyl-D,L-phenylalanine-β-naphthyl ester, stain: fast blue B salt). 1, 2, and 4: extract from spontaneous hay molding; 7, 8: extract from good hay; Met: metabolic products of M. faeni culture on synthetic medium; Exp: experimental hay molding with M. faeni.

**Table 1.** Quantitative analysis of protein content and enzymatic activity (chymotrypsin-like) of spontaneous and artificial moldy hay, good hay, and metabolic products from M. faeni culture

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Protein (μg of lyophilized extract)</th>
<th>Enzyme activity (U) (of β-naphthol per mg of lyophilized extract)</th>
<th>Sp act (U/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moldy hay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>0.80</td>
<td>80.0</td>
</tr>
<tr>
<td>2</td>
<td>54</td>
<td>0.90</td>
<td>16.6</td>
</tr>
<tr>
<td>3</td>
<td>41</td>
<td>1.30</td>
<td>31.7</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>10.40</td>
<td>260.0</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>0.78</td>
<td>48.8</td>
</tr>
<tr>
<td>6</td>
<td>41</td>
<td>0.78</td>
<td>19.0</td>
</tr>
<tr>
<td>Good hay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>44</td>
<td>0.20</td>
<td>4.5</td>
</tr>
<tr>
<td>8</td>
<td>45</td>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>Metabolic products (synthetic medium)</td>
<td>58</td>
<td>49.5</td>
<td>853.4</td>
</tr>
<tr>
<td>Experimental hay</td>
<td>Culture</td>
<td>35</td>
<td>1,600.0</td>
</tr>
</tbody>
</table>

ide gel and hyperimmune serum of a rabbit immunized with *M. faeni* is shown in Fig. 2. This picture shows that at least two distinct fractions with chymotryptic activity seem to be antigenically related to those of *M. faeni*. We observed similar reactions with other moldy hay samples, however with different patterns. In sample 1 for example, the slow-migrating (cathodic) band (Fig. 1) gave a line of precipitation against *M. faeni* antiserum. The more cathodic arc in Fig. 2 from sample 4 was not present in the other samples. Thus, we were able to demonstrate with this technique the identity of at least three fractions with chymotryptic activity in our moldy hay samples with that of *M. faeni*. The good hay sample, including sample 7, which had a band (Fig. 1), did not show any reaction against *M. faeni* antiserum.

**Immunoelectrophoresis.** A better-known demonstrative approach to the identification of the enzymes is the direct electrophoresis of the extracts in agar gel with a subsequent reaction
against *M. faeni* rabbit immune serum. Figure 3 illustrates the distribution of the chymotrypsin-like enzymes in the hay samples which are common to *M. faeni*, although they present different patterns. Samples 1, 2, 3, 5, and 6 show the same pattern as illustrated by samples 1 and 2 in Fig. 3, namely, two superimposed anodic lines of precipitation. The other pattern is illustrated by sample 4 (Fig. 3) with two arcs electrophoretically well separated. It is interesting to note that the artificial *M. faeni* hay culture and to some extent the metabolic products from the synthetic medium culture (Fig. 3) show a combination of the two patterns found in moldy hay. Again, the two samples of good hay did not harbor any chymotrypsin fraction identical to *M. faeni*.

**DISCUSSION**

The hypothesis that moldy hay as a whole represents the main source of allergens in allergic manifestations of the respiratory tract is not new. The pioneer work of Pepys et al. (20) and the interesting observations of Parish (17) provided convincing evidence for support of the hypothesis. This served as the basis of our interest in acquiring more precise knowledge about the nature of the antigens found in moldy hay.

We were surprised indeed to find that the total protein content of both moldy and good hay extracts obtained by the same method showed no apparent differences. However, since we were able to detect the presence of protein bands with enzymatic activity in the moldy hay, we are inclined to believe that the protein pattern of moldy hay may be qualitatively different from that of good hay extracts. This may be the result of degradation of the hay which serves as a growth substrate, by the invaders, with the subsequent release of new proteins.

This hypothesis was confirmed by the demonstration of the presence of extracellular enzymes from *M. faeni*, the main organism in our moldy samples. We detected such enzymes with chymotrypsin activity and which had antigenic identity with those from *M. faeni* in all the moldy samples. The distribution of these enzymes differed in each moldy sample examined. In all moldy samples two broad bands with strong activity were present in the disc electropherogram (Fig. 1). Other bands more cathodic

**Fig. 2.** Identification of chymotrypsin-like enzymes in disc immunelectrophoresis. Moldy hay extract 4. MF, *Micropolyspora faeni* antiserum (enzymatic activity and staining as in Fig. 1).**

**Fig. 3.** Identification of chymotrypsin-like enzymes in immunelectrophoresis. 1, 2, 4: extract from spontaneous hay molding; 7: extract from good hay sample; Exp: experimental hay molding with *M. faeni*; Met: metabolic products of *M. faeni* culture on synthetic medium; MF, *Micropolyspora faeni* antiserum (enzymatic activity and staining as in Fig. 1).
in position were found to occur in some samples (samples 1 and 4). All three bands showed an antigenic relationship to *M. faeni*. In one good hay extract (sample 7), we were surprised to observe one band with rather weak activity and also without any cross-reactivity for the enzymes of *M. faeni*. This suggests another origin such as the plant itself or perhaps from a related saprophyte. The other good hay sample (sample 8) did not show any band with enzymatic activity.

The quantitative estimation of chymotryptic activity (Table 1) shows extreme variations in the amounts of these chymotryptic extracellular enzymes of *M. faeni* present in the different moldy samples. This explains the differences in the organic quality of the samples, which probably are reflections of the degree of invasion by *M. faeni* and on the conditions of growth for this actinomycete. Optimal growth of *M. faeni* depends on a variety of factors such as the water content of hay which relates to self-generated heat in curing of the hay and most likely the nature of competitive flora. Such conditions vary widely in different natural molding processes and this explains the variations in the release of the metabolic products of *M. faeni*. Our artificially produced moldy hay (*M. faeni* on sterilized hay) showed qualitative data similar to the spontaneously molded samples, especially sample 1. However, the enzymatic activity in the artificial laboratory controlled hay culture was found to be from 6 to 100 times greater than that in the spontaneously molded hay samples.

To prove that the enzymes found in moldy hay are really extracellularly released during the growth of *M. faeni*, we compared our findings of moldy hay extracts with those of the metabolic products obtained from the synthetic medium after cultivating *M. faeni* and removal of all spores and mycelia from the medium. We succeeded in demonstrating that with a qualitatively good yield (Table 1) the pattern of production of chymotryptic enzymes was the same in culture medium as in moldy samples. These observations confirm the hypothesis that the enzymes believed to be associated with allergic alveolitis are contaminants released from the actinomycete during its growth metabolism.

The practical implications of such findings in the pathogenesis of respiratory distress in man and animal lead us to the following conclusions. There is strong evidence that the qualitative and quantitative compositions of the allergens differ in different moldy samples in spite of the predominance of *M. faeni* as the main molding agent. The quantitative presence of definite allergens could thus be of crucial moment to the onset of the allergic reactions. The constant presence of precipitins against enzymes with chymotryptic activity from *M. faeni* in patients affected with extrinsic allergic alveolitis (1, 26) suggests the important role of these enzymes, which appear to be good antigenic substances, as possible allergens. The enzymes released in moldy hay may be inhaled with the moldy dust and act as allergic immunogens either as surfactants or soluble antigens absorbed through the alveolar membranes into the circulation. All moldy samples which we examined contained such enzymes and all were involved in clinical cases of extrinsic allergic alveolitis in man or in cattle. We are now conducting further studies with the objective of purifying and characterizing immunogenic enzymes from *M. faeni*. Ultimately we hope to define the possible role which they play in an allergic alveolitis.

If these released enzymes retained their full enzymatic activity we should consider another possibility in the pathogenesis of allergic alveolitis, namely, the possible direct activity of such enzymes on the alveolar tissue leading to emphysema-like syndrome. Or these may be a pharmacological activity on the mucous membranes of the bronchi resulting in broncho-constriction and a decrease in tidal air. This has been observed in dogs and cats after experimental inhalation of chymotrypsin, trypsin, and other proteases (7, 24). Further investigations are urgently needed to determine the exact pharmacological or enzymatic role played by these exogenous enzymes on the respiratory tract and to determine their behavior against enzyme inhibitors of the host.

Our study shows the importance of characterization of allergens found in moldy hay for a better understanding of the pathogenesis and diagnosis of allergic respiratory distress in man and animals. Perhaps then we may appreciate the complexity of the pathogenesis in such respiratory diseases.

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LITERATURE CITED


