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The effect of *Aspergillus oryzae* fermentation extract on the anaerobic fungi *Neocallimastix frontalis* EB 188, *Piromyces communis* DC 193 and *Orpinomyces* ssp. RW 206: generalized effects and component analysis

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Abstract Three fungi *Neocallimastix frontalis* EB 188, *Piromyces communis* DC 193 and *Orpinomyces* ssp. RW 206, representing the predominant cultures isolated from cattle, were shown to respond to the addition of *Aspergillus oryzae* fermentation extract (i.e., Amaferm; BioZyme Inc., St. Joseph, Mo.) stimulation. Growth rates, protein and cellulase secretion and fungal mass production were all accelerated in the presence of the extract. Analysis of volatile fatty acids produced by these three species suggested that extract addition increased and altered gas production. Fractionation and preliminary analysis of the components present in the soluble extract, which stimulated the growth of the cellulolytic fungus *N. frontalis* EB 188, were also attempted. Soluble and filtered, sterilized extract was treated prior to use as a stimulant. Pretreatments included dialysis, ultraviolet irradiation, freeze thaw cycling, boiling, autoclaving, digestion with protease, autodigestion, organic extraction, decolorizing-carbon binding and polyethylene glycol concentration. Boiling, protease treatment, organic extraction, freeze thaw cycling and decolorizing-carbon binding reduced the ability of the extract to stimulate fungal cultures. Gel electrophoresis methods demonstrated that protein- and cellulase-secretion profiles were not identical in control and stimulated cultures. High-performance liquid chromatography methods allowed the separation of the extract into a limited number of ultraviolet-absorbing peaks, of which several stimulated the physiology of the fungus.

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Introduction

One of the more promising areas of animal nutrition research is in the area of feed supplementations. Directly fed microbials (e.g., Amaferm) have decreased heat stress, increased fiber conversion, enhanced animal well-being and increased milk production in ruminants (Campos-Montiel et al. 1991; Fondevila et al. 1990; Gomez-Alavcon et al. 1990; Kellems et al. 1990; Varel and Kreikemeier 1994). Most directly fed microbials can be administered for few pennies a day, are easily administered and stored, have been approved for use by (or ignored by) the FDA and USDA, are safe to use (Barbesgaard et al. 1992) and are commercially available worldwide. The biggest barrier to more extensive use of these supplements is their inconsistency. Laboratory studies have suggested that rumen bacteria possess accelerated cellulose-degrading activities (Beharka et al. 1991; Newbold et al. 1991) and more rapid lactate-uptake systems (Nisbet and Martin 1989) in the presence of soluble extracts of Amaferm. Although more than 100 university-based studies have been published describing animal responses to various directly fed microbials (see Martin and Nisbet 1992), their mode of action remains a mystery.

The formulation of second-generation directly fed microbials possessing a high predictive value will rationally occur only after we know precisely how those of merit work. The ability of Amaferm powder to stimulate the physiology of an anaerobic fungus has been presented in a companion paper. Our *in vitro* tester system using fungi measures physiological parameters such as secretion of cellulases, and we believe it may be possible inexpensively and rapidly to gauge the impact such products will have on animal performance. The tester system may also provide a way to identify the active component(s) present and discern their mode of action. Studies here prove that extract can stimulate the major anaerobic fungi in cattle. Certain physical

characteristics of the stimulation component or components are described and evidence is provided demonstrating that the soluble components of Amaferm can be fractionated using chromatography procedures.

Materials and methods

Chemicals

All chemicals, unless otherwise mentioned, were purchased either from Sigma Chemical Company (St. Louis, Mo.) or from U.S. Biochemicals (Cleveland, Ohio). All chemicals used were of analytical grade or the best grade available.

Culture and growth conditions

All anaerobic fungi were isolated from beef cattle, housed at Washington State University, Pullman, Wash. USA.

Neocallimastix frontalis EB 188 (ATCC 76100), *Piromyces communis* DC 193 and *Orpinomycin* ssp. RW 206 cultures were identified by published methods (Orpin 1977; Joblin 1981). The composition of the culture medium has been described previously (Barichievich and Calza 1990b; Calza 1991; Welch et al. 1996). Cultures were grown using standard anaerobic procedures (Hungate 1966) in either stoppered 10-ml test-tubes or 200-ml round-bottom flasks. Cellulose (Sigma Cell 100) was used as the carbon source.

Preparation of extracts

Amaferm was manufactured by BioZyme Inc. (St. Joseph, Mo.) and supplied as a dried powder. Soluble extracts of Amaferm were prepared by dissolving 5 g Amaferm into 100 ml culture medium lacking a carbon source (Barichievich and Calza 1990a). The resultant slurry was bubbled at room temperature using anaerobic CO_2 for approximately 60 min. Filtration first through a Whatman no. 1 filter-paper and then through a 0.22-μm sterilization filter (Nalgene) provided the sterile and soluble extract used in the stimulation experiments. Extract preparations were tested for sterility by plating on Luria broth plates after each pretreatment. The preparation was deemed sterile if microbial growth failed to develop after 2 days of incubation at 39°C. Extract was added to 7 μl/ml culture medium in all *N. frontalis* EB 188 cultures, unless otherwise noted. A single preparation of zoospores was used for any single experiment comparing extract stimulations (Welch et al. 1996).

Dialyzed soluble extract was prepared using Spectropore tubing with an 8-kDa cut-off and dialysis was against 3000 volumes of doubly distilled H_2O at 4–6°C for up to 48 h unless otherwise noted. Extract that was freeze/thaw cycled was prepared by using at least five cycles of rapid freezing (in liquid nitrogen) followed by rapid thawing (in a 39°C water bath). Such material was usually stored at −20°C for several days between cycles. Storage of pretreated extract at −20°C or −85°C was considered long-term after 30 days. Boiled extract was prepared by placing samples in boiling (98°C) H_2O for 20 min. Autoclaved extract was prepared by autoclaving at 121°C and 103 kPa (15 lb in^{-2}) for 15 min. Ultraviolet (UV)-irradiated extract was prepared by exposing the extract in plastic plates without lid to a source of UV (low-pressure germicidal bulbs) generating a fluence rate of 0.722 mJ cm^{-2} s^{-1} (Calza and Schroeder 1982).

The exposure time was 10 min at room temperature with mixing. Organic extraction of sterile extract was accomplished by mixing with an equal volume of culture-medium-saturated chloroform for 10 min at room temperature. Phases were separated by centrifugation (5000 *g* for 5 min) and the aqueous layer was collected for use in experiments. Protease treatment of the extract was performed on extract dissolved in culture medium lacking a carbon source (at pH 6.55) by adding Pronase E (Sigma Chemical Co., St. Louis, Mo.) to 10 μg/ml and digesting at 37°C for 1 h. Under these conditions, the activity of Pronase E approximates the turnover rate advertised by the supplier. Heating to 80°C for 15 min was used to inactivate the Pronase E. Autodigestion of the extract was accomplished by placing soluble extract at 39°C for 48 h before using. Decolorizing-carbon binding of the extract was accomplished by adding 1% w/v activated charcoal to the extract, mixing for 2 h and filtering through first a sterile Whatman no. 1 filter-paper and finally a 0.22-μm filter (pulled by a vacuum). Polyethylene glycol concentration of the extract was accomplished by adding polyethylene glycol up to 30% (w/v), chilling to 4°C for 1 h and collecting the pellet by centrifuging at 10000 *g* for 30 min. The pellet was then resolubilized in an appropriate volume of culture medium lacking a carbon source. Extract preparations were tested for sterility as described above.

Assay methods

Methods for the analysis of culture mass and cellulases have been reported previously (Li and Calza 1991a,b; Tsai and Calza 1993). Protein in the culture mass and supernatants was measured by a dye-binding assay (Bradford 1976). Quantification of the remaining culture carbon source was by the anthrone assay (Hodge and Hofreiter 1962). Measurement of volatile fatty acids in the culture liquor using gas chromatography was performed, after acidification, by the methods of Holdeman et al. (1977).

All experiments were run at least in triplicate and all reported data points represent an average of at least three replications per assay. The standard errors of assays were less than 1.3%. Statistical methods were used to determine observational significance (SAS 1989). Values were considered statistically significant at P values below 0.05.

Analytical measurements

Secreted products from cultures of *N. frontalis* EB 188 were analyzed by polyacrylamide gel electrophoresis. The denaturing and nondenaturing conditions of the electrophoresis and the cellulase overlays (or zymograms) used have been described previously (Barichievich and Calza 1990b).

High-performance liquid chromatography (HPLC) was used to study the soluble extract (as prepared above) and was performed at room temperature (22°C) with a tertiary system equipped with a reverse-phase C18 column (Merck Inc., Germany). The elution solvent was isopropyl alcohol used at 0.5%–25% v/v. Sample elution was followed by UV absorption spectroscopy at 215 nm. Fractions were collected and assayed for their ability to stimulate *N. frontalis* EB 188 culture as described above.

Results

Experiments were carried out to test the extract stimulation phenomena in all the predominant anaerobic fungi of cattle. After 48 h of growth, *N. frontalis* EB 188, *P. communis* DC 193 and *Orpinomyces* ssp. RW 206 all showed an increase in the secretion of cellulases (except β-glucosidase) and in protein and culture mass production in the presence of extract (Table 1). Values above those of the controls were evident in all species by 48 h

of growth and increased with time throughout the sampling period.

The effect of the extract on the volatile fatty acids produced by each of the three fungal species is shown in Table 2. Statistically significant increases in the production of acetate and total volatile fatty acids (i.e., 19.2%–23.0%) were evident.

To identify the components within the soluble extract that mediate the stimulation in *N. frontalis* EB 188, experiments were set up to test extracts that had been pretreated. UV irradiation, dialysis, autoclaving and freeze/thawing failed to abolish the effect (Fig. 1). Most treatments slightly decreased the stimulation of cellulase (endoglucanase) but failed to reduce the effect to control values. All decreases (with the exception of those following boiling and freeze/thaw treatment) were statistically insignificant. Boiling the extract caused a statistically significant reduction ($P < 0.05$) in cellulase secretions and nearly abolished the effect on the secretion of protein. The amount of β-glucosidase secreted and culture mass produced in the cultures treated with frozen/thawed extract were similar to values for control cultures.

Additional pretreatments of the extract were tested for their effect on the secretion of cellulase by the culture. Organic extraction, protease treatment, long-term storage or freeze/thaw cycling and decolorizing-carbon binding all abolished (i.e., to within 5% of control values) the stimulation effect of the extract. Extract that had been stored for a long time caused a slight increase (7.2% above controls) in the secretion of cellulase but the values were not statistically significant. Untreated extract caused a 20.1% increase in cellulase in cultures inoculated with the same preparation of zoospores. Autodigestion or polyethylene glycol concentration failed to reduce the extract's ability to stimulate secretions and such cultures respectively showed increases in cellulase of 24.1% and 19.7% over control cultures. Polyethylene glycol itself, however, significantly slowed the growth of the fungus.

Dialysis experiments were studied more closely since a precipitate formed during the process. Dialysis for 20 h reduced the salt concentration to that of the dialysate. The precipitate within the bag was first evident at 20 h and had reached a maximum by 60 h. Such extract, clarified by centrifugation, was, however, completely effective in stimulating fungal cultures. The secretion of cellulase and protein equalled that of cultures treated with normal extract. Such extract, however, possessed only 37% of the protein originally dissolved in the preparation.

To characterize further the extract-caused stimulation of fungal physiology, gel electrophoresis was used to analyze secreted proteins and cellulases. Normal extract added to 7 µl/ml culture medium did

Table 1 Effects of extract on fungi

Fungus	Increase (%)			
	Endo-glucanase	Supernatant protein	Culture mass	β-Glucosidase
Neocallimastix ($n > 14$)	135.5	114.3	115.7	113.9
Piromyces ($n = 5$)	120.2	125.8	118.7	115.2
Orpinomyces ($n = 4$)	127.1	121.7	131.0	99.5*

Values are expressed as a percentage of the control
* Not significant

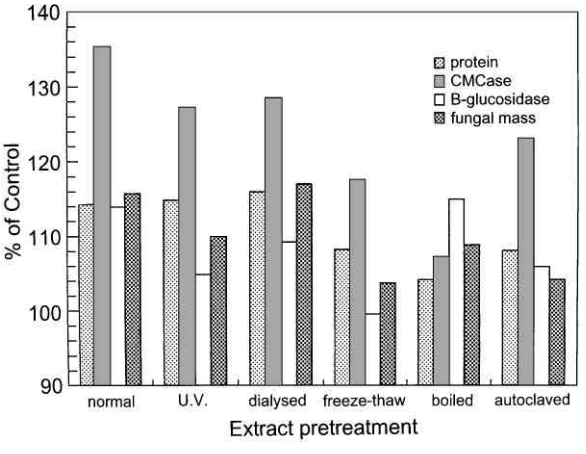

Fig. 1 Culture response after exposure to pretreated extract: cellulase (*horizontal shading*), β-glucosidase (*no shading*), protein (*gray shading*) secretion and culture mass production (*vertical shading*)

Table 2 Analysis of volatile fatty acids (VFA) of cultures. The acetate increase is the increase in acetate concentration above that of the control cultures. The total VFA includes acetate, propionate, butyrate, isobutyrate, valerate and isovalerate, and the VFA increase is the increase in total VFA concentration above that of the control cultures

Fungal strain	Treatment	Acetate (mmol/ml)	Acetate increase (%)	Total VFA (mmol/ml)	VFA increase (%)
EB 188	Control	6.94	–	7.01	–
	Extract	7.77	11.96	8.62	22.97
RW 206	Control	7.25	–	7.30	–
	Extract	8.22	13.38	8.70	19.18
DC 193	Control	6.95	–	7.38	–
	Extract	7.16	3.02	8.82	19.51

not contribute directly to the protein or cellulase banding pattern. Denaturing gels showed that mostly similar proteins were secreted in either control or extract-stimulated cultures. Proteins of molecular masses ranging from approximately 130 kDa to 20 kDa were present in both samples. Extract-treated cultures, however, did possess a 48-kDa and an 84-kDa protein in greater concentrations than in control cultures. Also, a 64-kDa protein was present in extract-treated samples but not in control samples. Nondenaturing gels coupled with *in situ* assays of cellulases (endoglucanases) allowed a comparison of the secreted cellulases in these cultures and these electrophoreses were performed. Once again, mostly similar patterns of cellulases were evident and activity bands with R_F values ranging from 0.05 to 0.77 were present in both samples. Bands at $R_F = 0.3$–0.35 and 0.77, present in extract-treated cultures, were, however, either absent or greatly decreased in control cultures.

Experiments were set up to determine whether the soluble extract of Amaferm could be fractionated into components possessing stimulatory activity using HPLC methods. Figure 2 shows an elution profile of extract passed through a reverse-phase column. The extract is separated into about 24 UV-absorbing peaks. The majority of the material was retained to varying extents by the column. Fractions from the HPLC column were collected and used to stimulate cultures of *N. frontalis* EB 188. Cellulase secreted into the supernatant is plotted against column elution time (Fig. 3). Analysis demonstrated that approximately 70% of the stimulating activity was eluted between 35 min and 56 min. One fraction (at 45 min) actually possessed 25% of the total stimulatory activity. Several other samples that contained UV-absorbing material were, however, unable to stimulate fungal cultures.

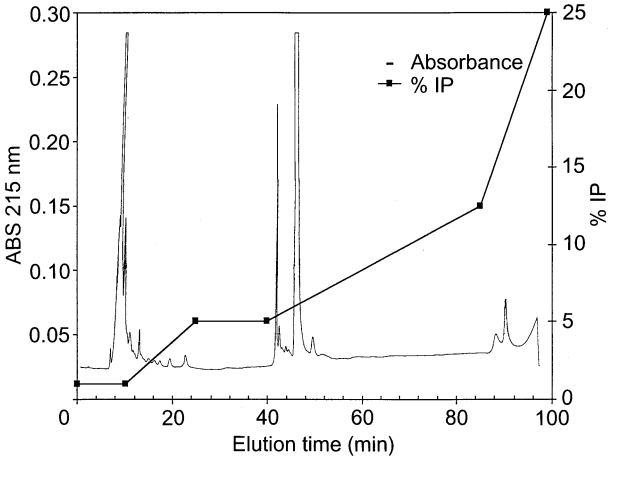

Fig. 2 HPLC of Amaferm extract. The absorbance (*ABS*) is plotted against the elution time. The percentage (v/v) of isopropanol (*IP*) is also plotted

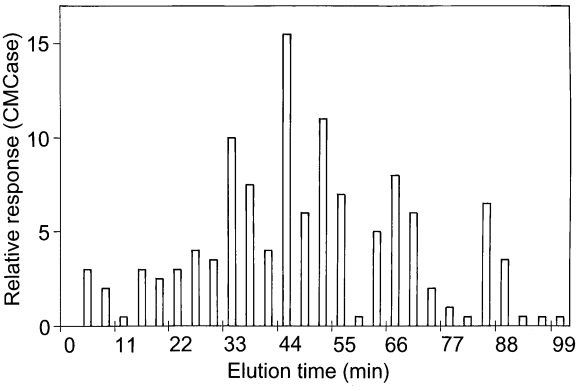

Fig. 3 Stimulation of the cultures by the HPLC fraction. The relative secretion of cellulase by the culture is plotted against the fraction's elution time. *CMCase* endoglucanase

Discussion

In the cellulolytic species of anaerobic fungi studied, the growth rate and the secretion of proteins and cellulases were accelerated in the presence of extract. Since these species represent the predominant fungi of cattle, it is likely that the response represents a generalized reaction of all the fungi in cattle. We have provided evidence demonstrating that slight differences in secretion patterns exist in extract-treated cultures. Stimulation may not simply be due to an acceleration of fungal physiology but also to an alteration in physiology. Extract addition also caused an increase in the production of volatile fatty acids and differences between fungal species were evident. Any impact on animal performance caused by such accelerations in fungal physiology cannot be directly extrapolated. Improvement in fiber conversion has, however, been demonstrated in sheep inoculated with laboratory strains of anaerobic fungi possessing atypical physiologies (Gordon and Philips 1993).

On the basis of this research, several characteristics of the components in soluble extracts of Amaferm that stimulate fungal physiology are evident. The presence of viable *A. oryzae* cells can be ruled out because sterile extract was used and several killing treatments (i.e., autoclaving, UV-irradiation, etc.) that would have inactivate tiny particles (e.g., viruses, etc.) failed to abolish the stimulation. The component(s) in the extract responsible for the stimulation are soluble. It is equivocal whether the components are heat-labile since boiling significantly decreased the stimulation ability, and yet autoclaving did not. We can not provide a plausible explanation for these findings. The stimulation component(s) are not enzymes since protease, cellulase, xylanase, pectinase, or lipase levels were extremely low (see Welch et al. 1996) and certain pretreatments would have inactivated such enzymes. Protease treatment and organic extraction, however, suggested that peptide-like molecules were important. The component(s) is not

a nutritional factor, such as a vitamin or carbon source, since the amounts of these components in Amaferm extract have been shown to be extremely low. The component(s) in the extract are not "salts" since extensive dialysis failed to diminish the stimulation ability. The molecular mass of the component(s) probably exceeds the 2-kDa cut-off of the membrane. HPLC experiments suggested that more than one component in the extract can accelerate the physiology of anaerobic fungi and that the component(s) may be UV-absorbing. This is the first report of such a finding. The identity of the component(s) in Amaferm responsible for the stimulation remains a mystery, but fractionation experiments may lead to their isolation.

The lack of heat lability of the extract recorded in this study contrasts with results of studies performed in rumen bacteria (Beharka et al. 1991; Newbold et al. 1991; Nisbet and Martin 1989). In those studies, autoclaving completely abolished the stimulatory efects caused by the extract *in vitro*. It remains possible that the effect mediated by the extract will vary with species. Consistent with earlier studies using bacteria, irradiation with short-wavelength light (i.e., UV) had no effect on the extract's ability to stimulate anaerobic fungi. This suggests that components responsible for the stimulation were not inactivated with 254-nm light.

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