EFFECT OF HOST ENZYME EXTRACTS ON THE ELECTROPHORETIC FORMS AND SPECIFIC INFECTIVITY OF COWPEA MOSAIC VIRUS

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INTRODUCTION

Two electrophoretic forms of cowpea mosaic virus (CPMV) have been characterized by Niblett and Semancik (12, 13). The slow-migrating form (S) predominates in early infection, while the fast-migrating form (F) predominates in late infection. Conversion of the S to the F form of CPMV results from the loss of amino acids from the viral protein coat and is accomplished *in vitro* with chymotrypsin or a mixture of carboxypeptidases A and B. The conversion of the S to the F form by these enzymes *in vitro* increases the specific infectivity of CPMV in spite of the declining quality of its RNA (13).

The *in vivo* conversion of the S form to the F form prompted Niblett and Semancik to speculate that host proteolytic enzymes were responsible (12). This report describes the partial purification of host enzyme extracts capable of converting the S form of CPMV to the F form *in vitro*, and the effect of cycloheximide (CH), an inhibitor of protein synthesis, on the normal *in vivo* conversion of S to F and its resultant change in specific infectivity.

MATERIALS AND METHODS

Virus culture

The Sb strain of CPMV described by Agrawal (1) was used throughout these investigations. Virus was purified from cowpea (Vigna unguiculata (L.) Walp. cv. Early Ramshorn) according to the procedure of Semancik and Bancroft (17). For production of CPMV, cowpeas were grown in growth chambers with 16-h photoperiods, 8-h dark periods, 30°C, 25% ± 5% relative humidity (RH), and an average light intensity of 30,000 lx at leaf height (determined by a Weston Illumination Meter, Model 756). Plants were inoculated by dusting with carborundum and rubbing with an inoculum made by homogenizing 1 g CPMV-infected plant tissue per 3 ml 0.02 M potassium phosphate buffer, pH 7.0 (KPO_4). Infected cowpeas were harvested and stored in plastic bags at -18°C for virus purification. CPMV concentrations were determined using $E_{260}^{0.1\%} = 8.0$ (12). Specific infectivity assays were performed on Pinto beans (Phaseolus vulgaris L. cv. Pinto) using eight half-leaves per treatment and a virus concentration of 1 µg/ml. Results are expressed as per cent of control, with the average number of local lesions per half-leaf for the control being reported.

Etiolated cowpea hypocotyls

Etiolated cowpea seedings, grown in the dark at 30°C and 85% RH, were inoculated with CPMV as described by Lockhart (10).

Electrophoresis

The electrophoretic forms of CPMV were separated by disc electrophoresis using a stacking gel and running buffer as described by Davis (5), except that 2.8% polyacrylamide gels polymerized with ammonium persulfate were used (J. S. Semancik, *personal communication*). Sample load was 50 µg CPMV/gel. Twelve gels were run simultaneously at 200 V for 75 min. The ratio of F form to S form (F/S ratio) was determined by planimetry of densitometer tracings of gels scanned at 260 mm with a Gilford 240 spectrophotometer and gel scanning carriage. Gels were preserved unstained in 3% acetic acid.

Enzyme purification

Plants for enzyme extraction included Early Ramshorn cowpeas, soybeans (*Glycine max* (L.) Merr. cv. Cutler), and Cherokee Wax beans (*P. vulgaris* L. Cherokee Wax). Plants were grown in growth chambers as described previously except that the average light intensity at leaf height was 38,000 lx. Primary leaves were homogenized for 2 min in a cold Waring Blendor at a ratio of 1 g tissue to 2 ml 0.1 M dibasic potassium phosphate with 0.5 M sodium chloride adjusted to pH 6.3 with acetic acid (KAS buffer). The homogenate was filtered through 2 layers of cheesecloth. The filtrate was heated to 55°C for 10 min, cooled rapidly on ice, and centrifuged at 48,000 *g* for 10 min. The supernatant was centrifuged 2 h at 147,000 *g* and this supernatant brought to 80% saturation with ammonium sulfate (6) and centrifuged

at 10,000 g for 10 min. Pellets were resuspended in 0.1 M dibasic potassium phosphate with 0.5 M sodium chloride adjusted to pH 7.0 with acetic acid (KS buffer) and dialysed in the same buffer overnight. The enzyme was sometimes further purified by chromatography on a Sephadex G-100 column (Pharmicia, Sweden).

Molecular weight determination

Molecular weights were estimated from a V_e/V_o plot against log molecular weight (21). Blue Dextran (V_o) (Pharmicia, Sweden), bovine serum albumin (BSA) (M.W. 67,000), and cytochrome C (Type III from horse heart, M.W. 12,400) (both obtained from Sigma Chemical Co.) were used as standards on a 2.5 x 39 cm bed of Sephadex G-100 equilibrated with 0.05 M KS buffer. Flow rate was 12.6 ml/h.

Proteolytic assays

(a) CPMV -- A typical assay for detecting enzymatic activity converting S to F consisted of 0.5 mg CPMV (F/S ratio $\leq$ 1.0) in 0.1 ml of 0.001 M KPO_4 buffer, 0.3 ml of 0.1 M KS buffer, and 0.1 ml of appropriate host extract. Reactions were performed in 10 ml ultracentrifuge tubes for 1 h at 37 °C and terminated by addition of 9 ml cold 0.01 M KPO_4 buffer and centrifugation for 90 min at 269,000 g. The pellet was resuspended in 0.001 M KPO_4 and the F/S ratio determined after disc electrophoresis. Eighty-five to ninety per cent of the virus added to the reaction was recovered. Boiled enzyme and buffer without host extracts served as controls.

(b) Non-specific protein substrates -- Casein (Nutritional Biochemicals Corp.) was boiled as a 1% (w/v) casein-0.1M KS buffer solution for 15 min, dialysed overnight in 0.1 M KS buffer, and stored at -18 °C until used. Hemoglobin (Type II, bovine crude powder, Sigma Chemical Co.) was prepared as a 2.5% (w/v) solution in 0.1 M KS buffer, dialysed overnight in this buffer, and stored at -18 °C until used. A 1% (w/v) solution of BSA (Fraction V powder, Sigma Chemical Co.) was prepared similarly.

A typical reaction contained 0.5 ml of one of the above substrates, 0.25 ml host extract, and 1.0 ml of 0.1 M KS buffer. The reaction was performed at 37 °C for 30 min in a 12 ml conical centrifuge tube and terminated by adding 0.2 ml 40% trichloroacetic acid (TCA). Tubes were placed in ice for 30 min and centrifuged 10 min at 1300 g. The supernatant was removed with a syringe and filtered using a Millipore Swinny Adapter with a 0.45 µ Millipore filter. The non-TCA precipitable materials in the filtered supernatant were determined as described by Lowry, et al. (11). Controls were treated with TCA at zero time.

Hide powder azure (Calbiochem) was used following the procedure of Rinderknecht, et al. (16) except 0.1 M KS buffer was used.

(c) Specific artificial substrates -- Benzoyl-DL-arginine-p-nitroanilide hydrochloride (BANA, Sigma Chemical Co.) was prepared by dissolving 6 mg in 1 ml dimethylsulfoxide and adjusting the volume to 100 ml with 0.1 M KS buffer. A typical reaction consisted of 2 ml BANA and 0.25 ml

host extract. After 30 min at 37 °C, the reaction was terminated by addition of 0.25 ml 40% TCA and set in ice for 30 min. After centrifuging for 10 min at 1300 g, the absorbance of the supernatant was determined at 410 nm. Using the E^{M}_{410}=8800 for p-nitroanilide (PNA) (3), the number of µmoles PNA per reaction was determined. Controls were treated with TCA at zero time.

Hippuryl-L-phenylalanine (HPA) and hippuryl-L-arginine (HA) (Schwarz/Mann) were used as described in the Worthington Manual (2) to measure activity of carboxypeptidases A and B, respectively.

Treatment of plants with cycloheximide

Primary leaves of cowpeas infected with CPMV were excised with a razor blade, rinsed in distilled water, blotted dry, and placed in petri dishes (15 x 100mm) containing either 20 ml deionized water or 20 ml of a solution containing 500 µg CH (Schwarz/Mann) per ml of deionized water. Solutions were changed daily. Starting at zero time, leaves were harvested at 24 h intervals and stored in plastic bags at -18 °C until the end of the experiment, when CPMV was purified from each sample. The F/S ratio and specific infectivity of the CPMV from each sample were determined.

Intact cowpeas were treated daily by spraying to runoff with either a solution containing 500 µg CH per ml deionized water or with deionized water. CPMV was purified from the primary leaves and quantitated as above. Some primary leaves were used for extraction of host enzymes as described previously.

RESULTS

Effect of host extracts on CPMV *in vitro*

Enzyme extracts capable of converting the S form of CPMV to the F form were extracted from both healthy and CPMV-infected cowpeas and Cherokee Wax beans. After incubation with these extracts, both the F/S ratio and the specific infectivity of the CPMV increased (Table 1). There appears to be no difference in the ability of extracts from either healthy or diseased tissues to convert the S form to the F form of CPMV *in vitro* with either cowpeas or Cherokee Wax beans. Boiled extracts had no effect on the F/S ratio or specific infectivity of the CPMV. Sephadex G-100 column chromatography was done in an effort to demonstrate differences in enzyme extracts from healthy and CPMV-infected tissues. Maximum virus-converting activity chromatographed at a molecular weight near 80,000 with both extracts (Fig. 1). This peak of virus-converting activity did not correlate with the peak of activity for any proteolytic substrate which was tried. No enzymatic activity was detected with either hide powder azure, BSA, HP, or HPA, while casein, hemoglobin, and BANA had enzymatic activity maxima at fractions causing little if any virus conversion.

TABLE 1

Effect of enzyme extracts from healthy and CPMV-infected cowpea and Cherokee Wax bean of F/S ratio and specific infectivity of CPMV

Source	F/S ratio[a]	Specific infectivity[a]
Expt. I[b]		
Healthy cowpea	226	174
Diseased cowpea	200	173
Healthy Cherokee Wax	221	145
Diseased Cherokee Wax	126	154
Expt. II[b]		
Healthy cowpea, undiluted	∞[c]	---
Healthy cowpea, 1/2 dilution	300	---
Healthy cowpea, 1/5 dilution	100	---
Diseased cowpea, undiluted	∞[c]	---
Diseased cowpea, 1/2 dilution	280	---
Diseased cowpea, 1/5 dilution	120	---

[a]Expressed as per cent of control. Boiled enzyme control for Expt. I and minus enzyme control for Expt. II had F/S ratios of 0.60 and 0.34, respectively. Specific infectivity controls produced an average of 80 local lesions/half-leaf. Reaction times for Expts. I and II were 1 h and 2 h, respectively. For Expt. I, enzyme was boiled 10 min, cooled on ice, centrifuged 10 min at 10,000 g, and supernatant used for assay.

[b]Age of plants was 15 d, diseased plants were 7 d post-inoculation.

[c]Only F form remaining.

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Fig. 1. Fractionation of enzymatic activity from healthy and diseased cowpeas capable of converting the slow- to the fast-migrating electrophoretic form of CPMV.

Two ml of each ammonium sulfate purified sample were applied to a Sephadex G-100 column, and 2.1 ml fractions were collected at an elution rate of 12.6 ml/h at 1-5 ^{o}C. The column, 2.5 x 39 cm, was equilibrated and eluted with 0.05 M KS buffer. Results are expressed as per cent change in F/S ratio of control. The F/S ratios for the minus enzyme control from the healthy and diseased fractions were 0.59 and 0.25, respectively. Assay time for healthy fractions was 2 1/2 h, for diseased fractions it was 3 h.

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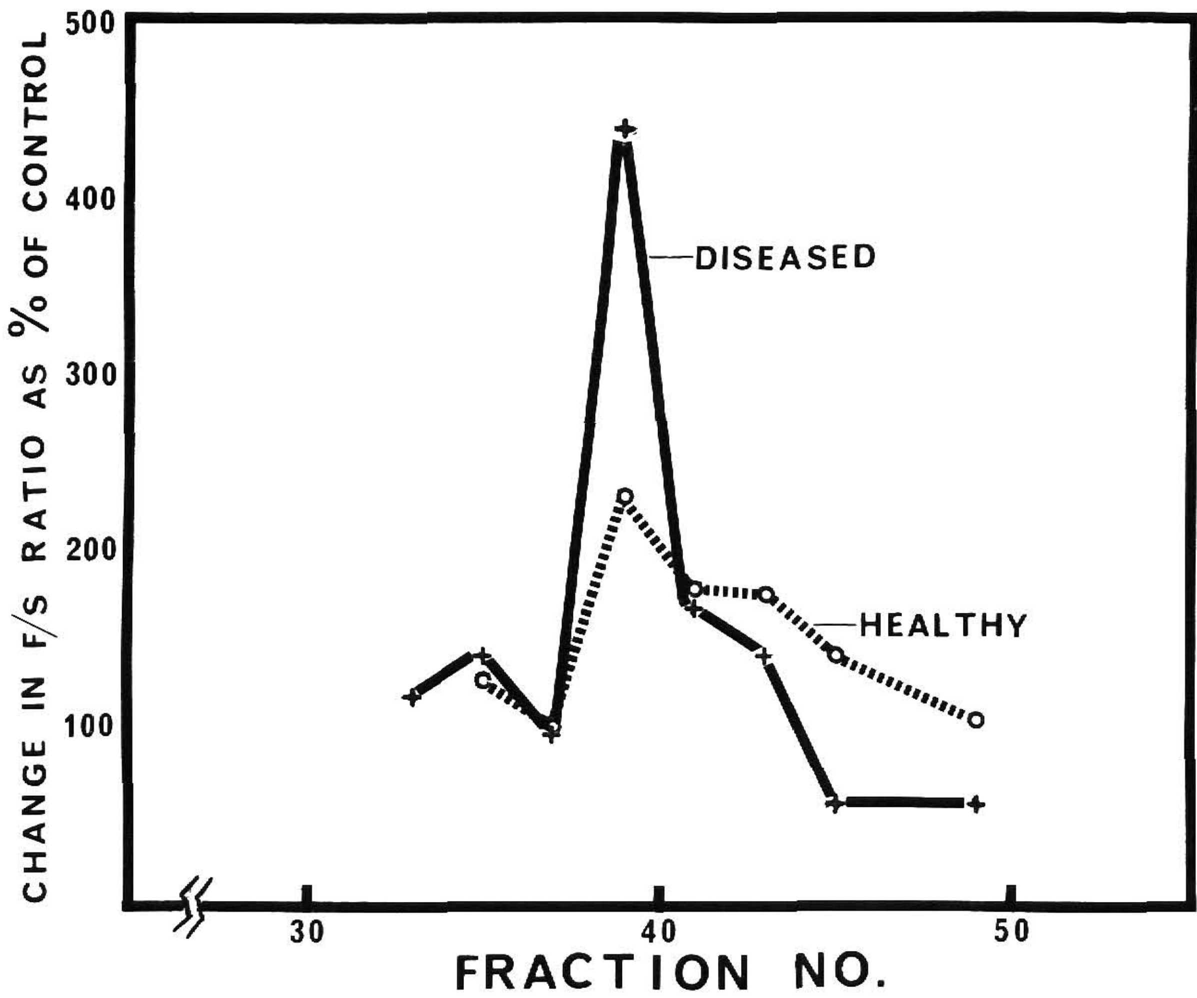
CHANGE IN F/S RATIO AS % OF CONTROL
500
400
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200
100
DISEASED
HEALTHY
30
40
50
FRACTION NO.

Effect of plant age on *in vivo* conversion of CPMV

To determine the effect of plant age on the CPMV S to F conversion *in vivo*, primary leaves of cowpeas from the same growth chamber were inoculated at three day intervals beginning three days after planting. Leaves were harvested 72 h after inoculation and stored at -18 °C. CPMV was purified from each sample and the F/S ratio determined. Results indicate that mature tissues possess greater ability to convert the electrophoretic forms of CPMV *in vivo* (Table 2).

Effect of CH on conversion of the electrophoretic forms of CPMV

The effect of CH on the normal *in vivo* conversion of S to F was determined by purifying CPMV from excised infected leaves floated on CH solution. The F/S ratio and specific infectivity of the purified CPMV were determined. In water-treated leaves only the F form was detectable and specific infectivity more than doubled after four days (Table 3). However, in CH-treated leaves the F/S ratio had only doubled and the specific infectivity increased only 21%. Similiar differences were obtained with primary leaves from intact cowpeas treated by spraying with either CH or water.

TABLE 2

Effect of plant age at inoculation on F/S ratio and yield of CPMV 72 h after inoculation

Age of plants at inoculation	F/S ratio	Yield[a]
3 days	0.03	2.5
6 days	0.02	5.0
9 days	0.09	15.0
12 days	0.24	16.0
15 days	0.31	15.0

[a]Yield expressed as mg CPMV/100 g of tissue

TABLE 3

Effect of cycloheximide on the *in vivo* conversion of CPMV in excised leaves at different ages of infection

Age of infection	Treatment	Yield[a]	F/S ratio	Specific infectivity[b]
4 d	0 time control	43	0.33	100
5 d	1[c] d water	23	0.74	120
5 d	1 d CH	13	0.37	90
6 d	2 d water	60	1.00	170
6 d	2 d CH	30	0.39	105
8 d	4 d water	96	∞[d]	223
8 d	4 d CH	21	0.65	121

[a]Yield expressed as mg CPMV/100 g of tissue.

[b]Expressed as per cent of 0 time control (60 local lesions per half leaf).

[c]Indicates number of days treated with water or cycloheximide (CH).

[d]Only F form present.

Attempts were made to extract virus-converting activity from CPMV-infected cowpeas sprayed with water or a CH solution. Activity could be extracted from control cowpeas sprayed with water but could not be extracted from the CH-treated plants. CPMV purified from samples of the above plants possessed a F/S ratio of 0.72 in the CH-treated plants while the F/S ratio was infinite or all F in the water treated plants. This experiment was performed seven days post-inoculation and agrees well with the data in Table 3.

Environmental effects

Niblett and Semancik reported a F/S ratio near 1.0 for CPMV at about 11 days post-inoculation (12). However, under local greenhouse conditions it was found that this was variable. A F/S ratio near 1.0 was obtained in summer months after 4-5 days, but 14-16 days were required during the winter months. Enzymatic activity capable of *in vitro* conversion of CPMV could not be extracted from greenhouse plants when 14-16 days were required to obtain a F/S ratio of 1.0 for CPMV. Therefore, plants used for CPMV and enzyme production were grown in growth chambers to eliminate this variability. Data in Table 4 indicate the relationship between light intensity and the S to F conversion of CPMV. Host enzymatic activity capable of this conversion could be extracted only from plants grown in growth chambers with a light intensity of 38,000 lx or greater or from plants grown in the greenhouse during the summer months. Although the *in vivo* S to F

conversion of CPMV occurs at lower light intensities, the host enzyme activity catalyzing this conversion could not be extracted from these plants.

Attempts were made to isolate virus-converting activity from etiolated cowpea hypocotyls grown in darkness and from soybeans grown under 38,000 lx. No activity could be isolated in either case. CPMV purified from the hypocotyls and soybeans possessed F/S ratios of 0.02 and 0.10, respectively, 8-10 days post-inoculation. This suggests that high light intensity is required for enzymatic activity in cowpea and that little enzymatic activity occurs in soybeans.

TABLE 4

Effect of light intensity on F/S ratio of CPMV from cowpeas

Days post-inoculation	Light intensity[a]	F/S ratio
4	38,000	1.00
7	30,000	0.75
8	30,000	1.05
9	28,500	0.80

[a]Light intensity expressed in lx is the average of 5 readings made at leaf height.

DISCUSSION

Host enzymatic activity capable of converting the S form of CPMV to the F form was extracted from both healthy and CPMV-infected cowpeas and Cherokee Wax beans. The estimated molecular weight of the virus-converting activity is near 80,000 for extracts from both healthy and diseased cowpeas. Plant proteolytic enzymes of this size are not unusual. Wells (20) characterized a carboxypeptidase-like enzyme with a molecular weight near 120,000 from French beans (cv. Prince). Racusen and Foote (14) reported a non-specific protease of near 100,000 molecular weight from Pencil Pod Wax beans. Zuber (22) has estimated the molecular weight by gel filtration of carboxypeptidase C from citrus peelings to be near 126,000.

The host virus-converting activity appears to be either unstable in dilute solutions, present in small amounts, or both. A 1/5 dilution of the ammonium sulfate extract has little activity on CPMV (Table 1). This activity can not be extracted from soybeans, etiolated cowpea hypocotyls, or from cowpeas grown under low light intensities. All contain lower amounts of the activity as indicated by the slow rate of F/S increase of CPMV with increasing age of infection.

CH, an inhibitor of protein synthesis (9), blocks the normal _in vivo_ increase in F/S ratio of CPMV with increasing age of infection. Host enzymatic activity capable of S to F conversion of CPMV could not be

extracted from cowpeas treated with CH. CH may inhibit the synthesis of the host enzymatic activity which converts CPMV and thus prevent the normal S to F conversion of CPMV with increasing age of infection. Alternatively, this inhibition by CH may be due to a more general disruption of cell metabolism rather than a specific inhibition of protein synthesis (7).

More than one enzyme may be involved in the S to F conversion of CPMV. The fraction from the G-100 column with the maximum virus-converting activity could be located at the point of overlap between two different enzymes essential for conversion. The molecular weight estimate of 80,000 would then be meaningless. Niblett and Semancik (12, 13) proposed that the *in vitro* conversion of CPMV was due to the removal of seven amino acids from the protein subunits of the intact S form. Removal of five neutral amino acids and two adjacent basic amino acids (Arg & Lys) converted S to F. A single enzyme possessing trypsin-like or carboxypeptidase A-like activity could not convert CPMV without the aid of an additional enzyme to subsequently hydrolyze the carboxyl terminal basic amino acids exposed by the trypsin- or carboxypeptidase A-like activities.

Current results (Table 1) indicate no consistent qualitative or quantitative difference in the enzymatic activity extracted from healthy or diseased plants, although this area needs further study. A less time-consuming assay of virus-converting ability is needed to facilitate this work.

There are several reports of the action of plant proteases on plant viruses (8, 15, 18, 19, 20). The action of proteases on plant viruses can cause electrophoretic and serological differences (4). In the case of CPMV, the use of proteolytic enzymes *in vitro* affected the electrophoretic properties and increased specific infectivity even though the RNA from the F form is less infectious and more polydisperse than RNA from the S form (13). To my knowledge, there has been no report of host enzymatic activity effecting the specific infectivity of a plant virus or comparing the virus-converting activity extracted from healthy and diseased plants. The host enzymatic activity involved here is unique in that the virus is rendered more infective.

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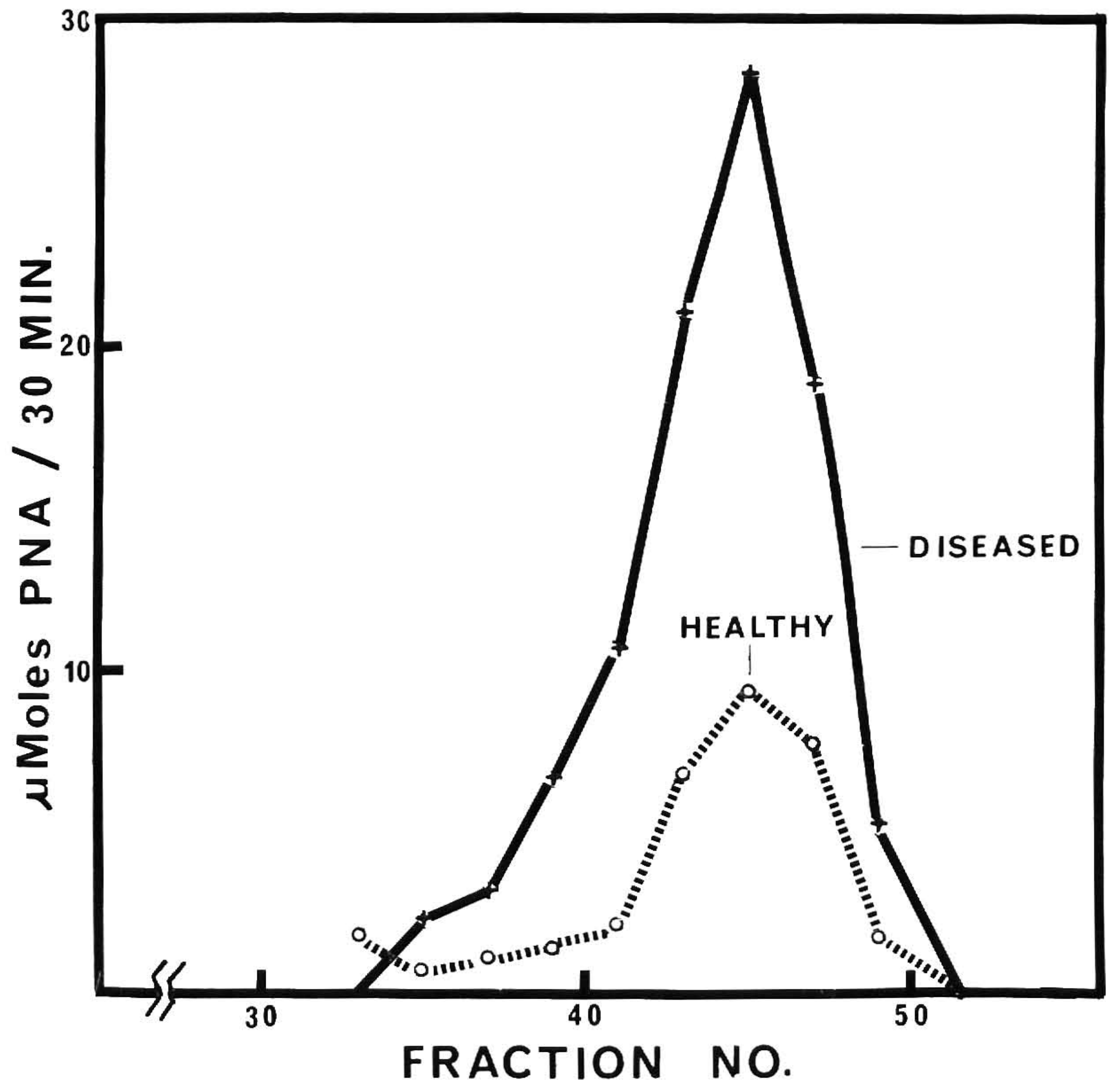
APPENDIX

Fig. 1. Fractionation of enzymatic activity from healthy and diseased cowpeas capable of hydrolyzing *p*-nitroanilide (PNA) from BANA.

Two ml of each ammonium sulfate sample were applied to a Sephadex G-100 column, and 2.1 ml fractions were collected at an elution rate of 12.6 ml/h at 1-5 ^{o}C. The column, 2.5 x 39 cm, was equilibrated and eluted with 0.05 M KS buffer. Activity is expressed as μMoles PNA hydrolyzed per 30 min reaction at 37 ^{o}C.



30
20
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μMoles PNA / 30 MIN.
DISEASED
HEALTHY
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FRACTION NO.

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ABSTRACT

Enzymatic activity capable of converting the slow-migrating electrophoretic form (S) of cowpea mosaic virus (CPMV) to the fast-migrating form (F) *in vitro* was extracted from both healthy and diseased cowpeas and Cherokee Wax beans. This conversion increased both the specific infectivity and the F/S ratio of CPMV. The virus-converting activity could not be extracted from cowpeas grown in growth chambers under low light intensity or under winter greenhouse conditions, from soybeans, or from etiolated cowpea hypocotyls. Maximum virus-converting activity from both healthy and diseased cowpeas chromatographed on a Sephadex G-100 column at a molecular weight near 80,000. No difference in virus-converting activity was detected between extracts from healthy and diseased cowpeas. The virus-converting activity was greater in more mature tissues.

Floating of excised CPMV-infected cowpea leaves in or spraying intact CPMV-infected cowpeas to runoff with a 500 μg/ml solution of cycloheximide inhibited the normal *in vivo* increases in both specific infectivity and F/S ratio of CPMV. Enzymatic activity causing *in vitro* S to F conversion of CPMV could not be extracted from intact cowpeas treated with cycloheximide. The S to F conversion of the electrophoretic forms of CPMV is apparently due to one or more host proteolytic enzymes.