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Appearance of a Host Protein in Cucumber Plants Infected with Viruses, Bacteria and Fungi

C. GESSLER[1] AND J. KUĆ

Department of Plant Pathology, University of Kentucky, Lexington, Kentucky 40546, U.S.A.

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ABSTRACT

Electrophoretic analyses of extracts of cucumber leaves infected with *Colletotrichum lagenarium*, *Fusarium oxysporum* f. sp. *cucumerinum*, *Pseudomonas lachrymans*, *Erwinia tracheiphila*, tobacco necrosis virus or cucumber mosaic virus revealed the presence of a protein band with an R_F value of 0·55–0·60 (based on mobility of bromophenol blue) on 10% polyacrylamide gel. This band was not evident in extracts of healthy or mechanically wounded leaves. The protein was not detected in uninfected leaves of infected plants, but it was detected in similar amounts in infected leaves and in secondarily challenged leaves of infected plants even though symptoms were not apparent on the latter. The protein had a molecular weight of approximately 16 000 d, was adsorbed on DEAE-cellulose, did not react with Schiff's reagent, and did not have ribonuclease activity. When injected into cucumber leaves, it did not inhibit germination of conidia of *C. lagenarium* or induce resistance against disease caused by the fungus.

INTRODUCTION

Four bands of host protein were detected electrophoretically in leaf extracts of *Nicotiana tabacum* L. cv Samsun, Samsun NN′ and Xanthi-nc tobacco, infected with tobacco necrosis virus (TNV) or, for the latter two varieties only, tobacco mosaic virus (TMV), but were not detected in extracts of leaves of uninfected plants (Gianinazzi, Martin, and Vallee, 1970; Van Loon, 1975; Van Loon, 1976; Van Loon and Van Kammen, 1968; Van Loon and Van Kammen, 1970; Rohloff and Lerch, 1977). The proteins were accumulated in tissue surrounding the lesions, and locally acquired resistance was developed concomitantly with the appearance of these proteins (Van Loon, 1975). New proteins were also observed in tobacco after infection with a number of systemic viruses (Van Loon, 1975), treatment with polyacrylic acid (Gianinazzi and Kassanis, 1974), or extracts of *Nocardia asteroides* (Gianinazzi and Martin, 1975). The infections and treatments conferred resistance to TMV (Gianinazzi and Kassanis, 1974; Gianinazzi and Martin, 1975; Gianinazzi *et al.*, 1970; Kassanis, Gianinazzi, and White, 1971). Kassanis and White (1974) inhibited the induction of local resistance to TMV and the formation of the new protein in tobacco plants by treatment with actinomycin D. Two of the protein bands were detected in extracts of uninoculated leaves of infected plants (Van Loon and Van Kammen, 1970; Rohloff and Lerch, 1977). Recently, Ahl, Benjama, Samson, and Gianinazzi (1980) reported that infection with *Pseudomonas syringae* induced the accumulation of the same proteins in tobacco as did infection with virus. Ziemiecki and Wood (1975*a*) reported changes in the pattern of soluble proteins in cucumber cotyledons after infection with cucumber mosaic virus

[1] Present address: Institut für Phytomedizin, Eidgenössische Technische Hochschule, Zurich, Switzerland.

(CMV). Two strains of the virus induced quantitative rather than qualitative changes in the protein pattern on polyacrylamide gels. The major changes appeared as an increase of the protein band at R_F 0·89–0·90 in 7% gels. Tas and Peters (1977) reported a protein in extracts from cucumber cotyledons infected with tomato spotted wilt virus (TSMV) or with CMV that was not evident in uninfected cucumber. They observed a close correspondence between the amount of the new protein (called E_1) and the severity of symptom expression. More recently, Andebrhan, Coutts, Wagih, and Wood (1980) reported that infection of cucumber foliage with *Colletotrichum lagenarium* or TMV also caused the accumulation of a new electrophoretically-detected protein. Actinomycin D applied prior to infection reduced the accumulation of the protein.

This paper reports on the observation of a similar, if not identical, protein in cucumber tissue infected with various pathogens and its relation to induced resistance as described by Kuć and his collaborators (Caruso and Kuć, 1979; Jenns and Kuć, 1977; Kuć, Shockley and Kearney, 1975).

MATERIALS AND METHODS

Maintenance and inoculation procedures were as described for *Colletotrichum lagenarium* (Pass.) Ell. & Halst race 1 (Kuć *et al.*, 1975), *Pseudomonas lachrymans* (Smith & Bryan) Carsner (Caruso and Kuć, 1979) and tobacco necrosis virus (Jenns and Kuć, 1977). *Fusarium oxysporum* f. sp. *cucumerinum* Owen was cultured on 2% malt agar. Plants infected with *Erwinia tracheiphila* (Smith) Bergey and CMV were supplied by G. Bergstrom.

Except as noted, the cucumber cultivar Marketer was used in all experiments. Systemic resistance was induced by inoculating the first fully expanded true leaf (leaf 1, inducer inoculation) with 30 5 μl drops of a conidial suspension of *C. lagenarium* (10^5 conidia ml^{-1}). The leaf above (leaf 2) was inoculated 7 d later (challenge inoculation) with the same concentration of conidia. Leaves were injured by applying dry ice to 30 sites on leaf 1 or by rubbing leaf 1 with carborundum.

Preparation of extracts

Leaves were homogenized in cold (0–2 °C) McIlvaine buffer pH 2·8 (buffer A, 1 g fr. wt./ml of buffer). The homogenates were centrifuged at 20 000 *g*, frozen for storage, and again centrifuged before use (crude extracts).

Electrophoretic analyses

Extracts or fractions were analysed, unless mentioned otherwise, at room temperature by disc-electrophoresis in 10% polyacrylamide gels using the system of Davis (1964) without spacer gel in tubes 4 mm × 70 mm. The gels were stained for 2 h in Coomassie brilliant blue G. The relative mobility of the protein bands (R_F) was expressed, taking as 1·0 the distance travelled by bromophenol blue. Flat-bed isoelectric focusing in thin layer of polyacrylamide gels was made with a final pH range of 3·5–9·5 (procedure after application note 250 LKB). Staining for Glycoproteins was made with Periodic acid-Schiff reagent (Matthieu and Quarles, 1973).

Molecular weight determination

The molecular weight was determined following the methods of Weber and Osborn (1969). The SDS-Page standard kit from Bio-Rad Corp. was used as a source of standards. It contained Lysozyme (14 300 d), soybean trypsin inhibitor (21 000 d), carbonic anhydrase (30 000 d), ovalbumin (43 000 d), bovine serum albumin (68 000 d) and phosphorylase b (94 000 d).

Partial purification

The crude extracts were adjusted to pH 3·5 with dilute hydrochloric acid and the precipitate was centrifuged and discarded. The resulting supernatant was pre-purified on a Sephadex G-25 column (2·5 cm × 30 cm) equilibrated with McIlvaine buffer pH 6·5 containing 0·3% 2-mercaptoethanol (buffer B). The protein fractions eluted with the void volume were pooled, dialyzed against Tris-HCl buffer (50 mM) containing 1 mM EDTA, 0·3% 2-mercaptoethanol (pH 8·0) (buffer C), loaded on a DEAE-cellulose column (1·5 cm × 10 cm), and eluted with buffer C and varying concentrations of NaCl.

Biological assays

Crude extracts from infected leaves and extracts from healthy leaves were used as liquid media for germination tests with *C. lagenarium*. One drop of the extract was mixed with one drop of conidial suspension (5×10^5 conidia ml^{-1}). The same assay was used with the partially purified protein fraction from the DEAE-cellulose column.

Crude extracts (1 g fresh leaf tissue 1 ml^{-1} extract) and partially purified protein fractions (approximately 0·01 mg protein ml^{-1} after Bradford, 1976) were injected into 5 sites (approximately 10 μl/site) of cotyledons and leaf 1 of cucumber plants, and 4 d later, leaf 2 was challenged with *C. lagenarium*. Partially purified protein fractions were tested for ribonuclease activity using an acridine orange RNA complex as substrate (Chaplinski and Webster, 1973). As control, commercial RNAse was added at the beginning of the extraction and tested for retaining it's activity.

RESULTS

Extracts from leaves infected with any of the pathogens used showed the appearance of a strong band of protein with an R_F value (based on the mobility of bromophenol blue) of 0·55–0·60 on 10% polyacrylamide gels (Fig. 1). We will refer to this protein band as Protein E. Protein E was not apparent in extracts of uninfected plants, uninfected leaves from plants infected with *C. lagenarium* or TMV, nor uninfected leaves from plants infected with the systemic pathogens *E. tracheiphila*, *F. oxysporum* f. sp. *cucumerinum* or CMV. Protein E

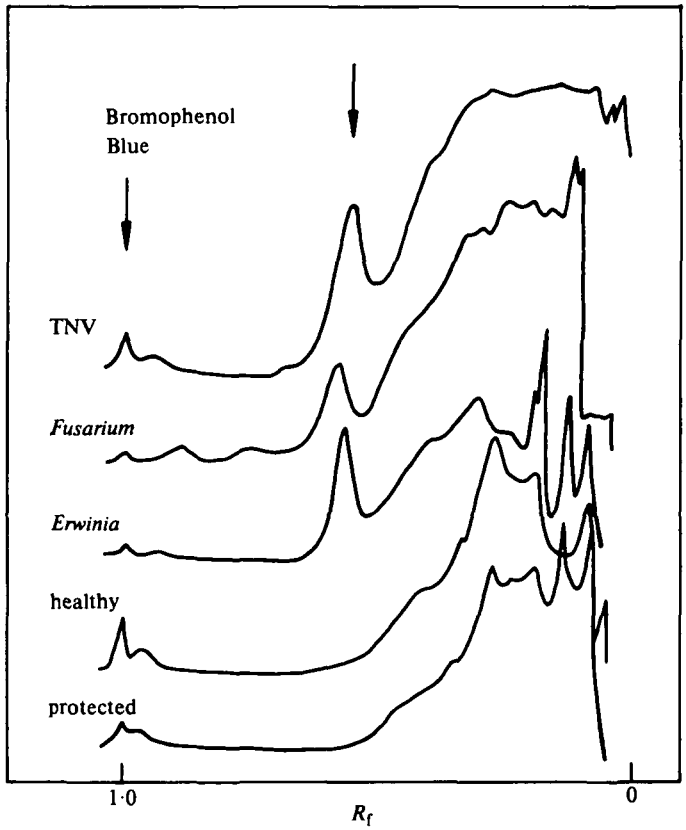

FIG. 1. Densitometer tracing of the electrophoretic patterns of extracts of leaves of cucumber cv. Marketer: uninfected (healthy) plants, uninfected leaves of *C. lagenarium* infected plants (protected) and leaves infected by TNV, *F. oxysporum* f. sp. *cucumerinum* or *E. tracheiphila*. Electrophoresis was conducted in 10% polyacrylamide gels.

was apparent in extracts of *C. lagenarium*-infected leaves from 9 cucumber cultivars (Table 1). In leaves infected with *C. lagenarium* the relative amount of protein E was proportional to the number of drops of inoculum and therefore also to the number of lesions formed. The protein was not detected in uninfected leaves above the inducer leaf which was infected with *C. lagenarium*, although these leaves were protected (80% reduction in lesion numbers) against disease caused by a subsequent challenge with the pathogen (Figs 1, 2).

TABLE 1. *Detection of protein E in extracts of infected leaves*

Cucumber cultivar	Pathogen	Band[c]
Marketer	water[a]	–
Marketer	injury[b]	–
Marketer	*C. lagenarium*	+
Marketer	*F. oxysporum* f. sp. *cucumerinum*	+
Marketer	*E. tracheiphila*	+
Marketer	*P. lachrymans*	+
Marketer	TNV	+
Marketer	CMV	+
Peppi	water	–
Peppi	*C. lagenarium*	+
SMR 58	water	–
SMR 58	*C. lagenarium*	+
Straight 8	water	–
Straight 8	*C. lagenarium*	+
Liberty	water	–
Liberty	*C. lagenarium*	+
Addis	water	–
Addis	*C. lagenarium*	+
Japanese pickling	water	–
Japanese pickling	*C. lagenarium*	+
Pointsette	water	–
Pointsette	*C. lagenarium*	+
Samson	water	–
Samson	*C. lagenarium*	+

[a] Uninoculated plants treated with water.
[b] Treatment with dry ice or rubbing with carborundum.
[c] + = band present, – = band not detected.

Extracts of challenged leaves from protected plants had approximately the same amount of protein E as extracts of challenged leaves from previously uninfected plants even though the leaves from these control plants had at least 10 times the number of lesions (Fig. 2). In one experiment, leaves from protected plants which showed no symptoms from a challenge inoculation with *C. lagenarium* (30 drops, 10^5 conidia ml^{-1}) on leaf 2, were extracted (Table 2). Unprotected plants inoculated on leaf 2, which had an average of $27{\cdot}4 \pm 2{\cdot}2$ lesions, and uninfected, unchallenged, healthy plants served as controls. For each extract, 3 leaves were used. Seventy-two hours after the inoculation with *C. lagenarium*, traces of protein E could be detected in extracts of all the inoculated leaves. From the 4th day to the 6th day a definite band was obtained with extracts of all infected leaves, although the protected challenged leaves were without symptoms 3, 4 and 5 d after challenge; 7 and 12 d after challenge protein

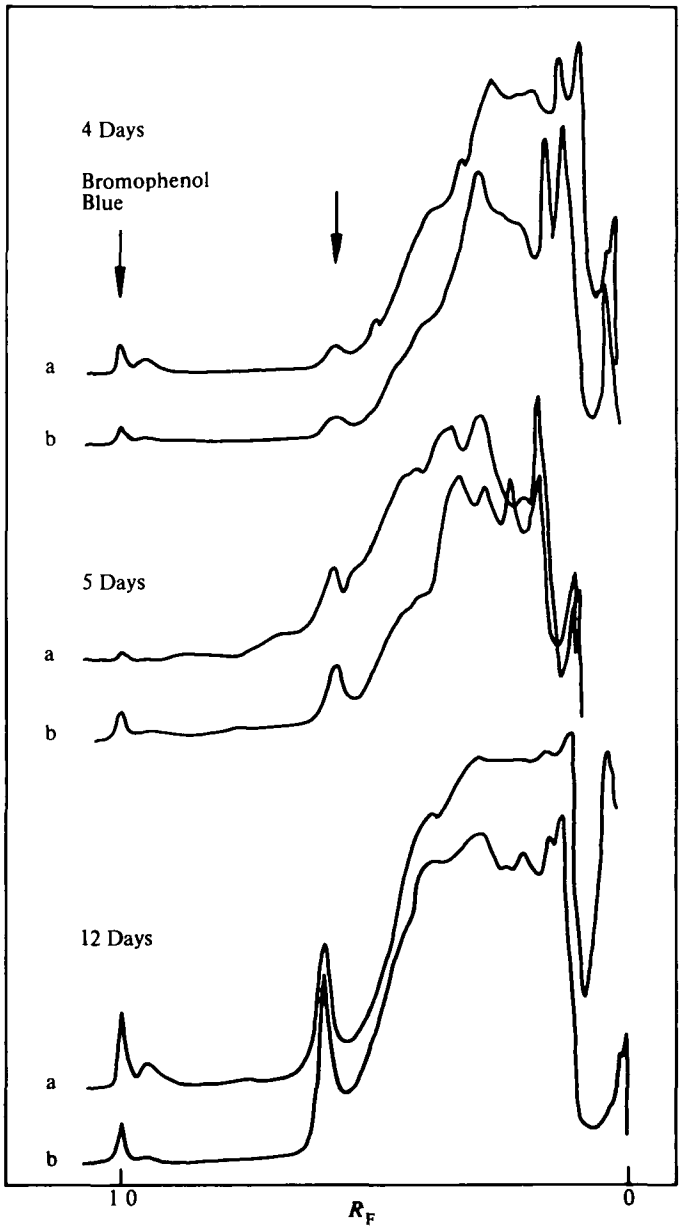

FIG. 2. Densitometer tracing of the electrophoretic patterns of extracts of leaves challenged with *C. lagenarium*. From (a) plants protected with prior inoculation on the lower leaf with *C. lagenarium* and (b) unprotected plants, at 4, 5 and 12 d after challenge.

E appeared as a strong band in all infected leaves though symptoms were not evident 12 d after challenge of protected plants. The band did not increase in intensity after day 7. Only in challenged leaves from protected plants were the concentrations of protein E not related to necrosis. In all other cases, chlorosis and the beginning of necrosis were associated with the presence of protein E. Mechanical injury (rubbing) or freezing part of the tissue with dry ice did not cause the appearance of protein E.

TABLE 2. *Detection of protein E as related to disease symptoms caused by* Colletotrichum lagenarium *in cucumber leaves*

Days after challenge	Protected plants symptoms[a]	Protein E[b]	Unprotected plants symptoms[a]	Protein E[b]
2	0	–	–	–
3	0	+	slight chlorotic lesions	+
4	0	++	28 clear chlorotic lesions	++
5	0	++	25 necrotic lesions	++
6	2·0	++	29 necrotic lesions	++
7	0·7	+++	28 necrotic lesions	+++
12	0	+++	27 necrotic lesions	+++

[a] Plants were protected by inoculating the first leaf with 30 5 μl drops of a conidial suspension of *C. lagenarium* (10^5 conidia ml^{-1}). Plants were challenged 7 d later on the second leaf as above. Symptoms were recorded 7 d after challenge as the number of lesions/leaf and appearance of lesions. Three plants were used per treatment.

[b] The amount of protein was visually estimated; –, absence of band; +, weak band; ++, clear band; +++, strong band.

Chemical properties of protein E

Protein E was partially purified by adjusting the pH of extracts from 5·6 to 3·5, centrifuging and discarding the pellet. The resulting solution produced less background on gels after electrophoresis and many bands of low R_F values diminished in intensity or

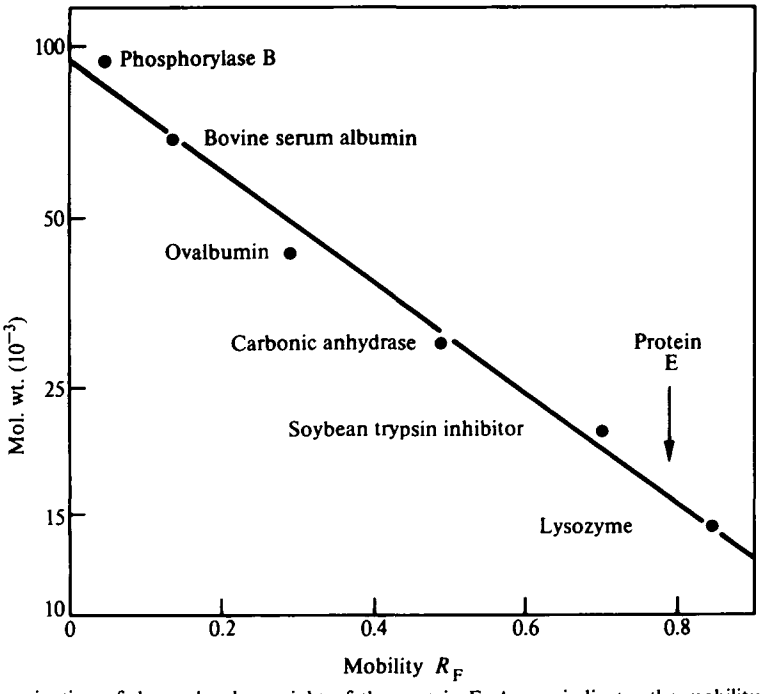

FIG. 3. Determination of the molecular weight of the protein E. Arrow indicates the mobility of the protein E. The extrapolated value for protein E is 16 100 d.

disappeared. The band at R_F 0·55–0·60 appeared unaffected by the procedure. Protein E appeared in the void volume after fractionation on a Sephadex G-25 column equilibrated with buffer A. After dialyzing against buffer C, the void volume fraction was loaded on a DEAE-cellulose column equilibrated with buffer C. Stepwise elution with varying NaCl concentrations revealed that protein E was eluted between 0·105 and 0·110 M NaCl as a broad peak.

Fractions from the DEAE-cellulose column containing protein E were denatured in SDS and the molecular weight of the single resulting band was determined as 16 100 d (Fig. 3). Flat-bed isoelectric focusing in polyacrylamide gel of purified protein E gave a single band at pH 4·5. On polyacrylamide gels, the band did not stain with Schiff's reagent. Ribonuclease activity was not associated with partially purified protein E. Commercial RNAse retained most of the activity during the purification procedure. Crude extracts from healthy and *C. lagenarium*-infected leaves had approximately equal RNAse activity.

Biological properties

The percentage germination of *C. lagenarium* conidia in crude extracts (equivalent of 0·5 g ml^{-1} fr. wt. of leaf tissue) from infected leaves and from healthy leaves were 69·6 ± 8·1% and 60·8 ± 4·2% respectively. The germination in 5% glucose was 69·4 ± 9·0%. In partially purified protein E fractions (equivalent amount of protein in 1 ml as in 1 g fresh leaf tissue) germination was 64·8 ± 6·3%.

Injection of protein E (same concentration as above) into cotyledons and first leaves did not induce protection in the upper leaves. The number of lesions developing on the second and the third leaves after the challenge inoculation was not different from the number on unprotected plants. In experiments performed concomitantly, plants with the cotyledons and leaf 1 infected with *C. lagenarium* were 80% protected, based on lesion numbers on unprotected plants.

DISCUSSION

Cucumber leaves and cotyledons were demonstrated to accumulate a protein after infection with a fungus or viruses (Andebrhan *et al.*, 1980; Tas and Peters, 1977; Ziemiecki and Wood, 1975*a*). In this report a protein was demonstrated to accumulate in leaves infected with fungi, bacteria or viruses. The organisms tested included local lesion and systemic pathogens. We conclude that the protein is of host origin and its accumulation does not depend upon the pathogen but rather upon a general host response to infection. Tas and Peters (1977) and Andebrhan *et al.* (1980) also reported that the protein which accumulates appears serologically unrelated to any of the coat proteins of the virus they tested. The protein detected in cucumber tissue of the Marketer variety infected with CMV (Ziemiecki and Wood, 1975*a*) was suggested to be possibly a coat-protein or coat-protein-related antigen (Ziemiecki and Wood, 1975*b*). It is unlikely that this suggestion is valid since the protein can be induced not only with the virus but also by different organisms. It seems that appearance of protein E is a general response to infection and common to all the tested cucumber varieties. A similar response is found in tobacco (Ahl *et al.*, 1980; Gianinazzi and Kassanis, 1974; Gianinazzi and Martin, 1975; Gianinazzi *et al.*, 1970) and it is not unexpected that further research will reveal similar proteins in other plants as a response to infection. From the properties on polyacrylamide gels it seems likely that the protein reported in this paper and in other reports are identical. The mol. wt. reported by Tas and Peters (1977) was estimated to be about 22 000 d by comparison to known proteins on polyacrylamide gels without SDS treatment. In our experiments the mol. wt. was determined by the method of Weber and Osborn (1969), with the use of SDS to be 16 100 d (Fig. 3). They found, contrary to us but in

accordance to Andebrhan *et al.* (1980), that the protein stains positive for carbohydrate. The difference in mol. wt. may be attributed to a carbohydrate moiety lost in our extraction method. Antoniw, Ritter, Pierpoint, and Van Loon (1980) found that three of the four b-proteins of tobacco plants (cv. Xanthi-nc) infected with TMV consisted each of a single polypeptide of similar mol. wt. of about 14 200 d but with different charge. Similarities of the protein E from cucumber and the certain b protein should not be excluded. At this time, reports of a direct role of the protein in restricting the growth of fungi, localization of virus, or inducing resistance are not available. Mercuric chloride sprays induced resistance in tobacco against TMV but did not induce the formation of the b proteins (Van Loon, 1975). We report that the protein accumulates in cucumber foliage infected with *F. oxysporum* f. sp. *cucumerinum*, a fungus which does not induce resistance against *C. lagenarium* (unpublished data). The presence of protein E in challenged, protected leaves, on the other hand, suggests that it is not only related to the formation of lesions, but is in some way related to the reaction of the plant to the presence of an infectious agent. When resistance induced with potato virus Y or polyacrylic acid is prevented in tobacco by Actinomycin D, the new proteins cannot be detected (Kassanis and White, 1974). Observations that RNAse activity is increased in virus-infected leaves has often been made (Randles, 1968; Wyen, Udvardy, Erdei, and Farkas, 1972). The level of RNAse also increased upon mechanical injury (Wyen *et al.*, 1972). Ziemiecki and Wood (1975*a*) suggested that protein E may be a RNAse but our tests did not confirm their suggestion. They also showed that iso-enzymes of peroxidase and polyphenoloxidase did not appear in the region of protein E on polyacrylamide gels (Wood and Barbara, 1971; Ziemiecki and Wood, 1975*a*). In similar tests, we confirmed this observation. The role of protein E is therefore still unclear.

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