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Effect of fructose on extracellular production of proteases by *Alternaria alternata* (Fr.) Keissl

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Abstract

Addition of fructose to the nutrient medium of *Alternaria alternata* (Fr.) Keissl has been found to give rise to higher protease production in the medium. It was seen that fructose neither stimulates the enzyme activity nor affects the secretion of enzyme in the extracellular fluid. These results indicate that fructose enhances the production of these enzymes.

Key words : Effect of fructose on protease, permeability, cell membrane.

1. Introduction

A number of compounds have been shown to affect microbial production of enzymes. Some of the compounds reduce production and some have been observed to enhance it. These compounds might exert their effects at different levels. Some of them might alter permeability characteristic of cell membrane resulting in more or less than normal secretion of enzymes in culture medium. Others could affect the actual biosynthesis of enzyme protein by influencing, for example, *m*RNA synthesis.

In earlier studies it was noted by the authors[1] that fructose and sucrose stimulate the production of proteases by *Alternaria alternata* (Fr.) Keissl. Stimulatory effect of sucrose is apparently due to the presence of fructose in it since glucose was ineffective. Stimulatory effect of fructose on apparent production of microbial proteases has been reported by several workers[2–5]. The present paper describes experiments carried out with a view to get an insight into the effect of fructose on protease production by *Alternaria alternata* (Fr.) Keissl.

2. Materials and methods

2.1 *Organism*

A. alternata (Fr.) Keissl isolated in this laboratory from infected mandarin oranges was maintained on potato dextrose agar slants. Spore suspension of the organism was prepared by scrapping the surface of culture in sterile distilled water and was adjusted to 2×10^6 spores/ml.

2.2 *Chemicals*

All chemicals used during the study were of analytical reagent grade. Rifampicin was a product of Ranbaxy Laboratories, New Delhi and chloromycetin was obtained from Parke Davis (India) Ltd., Bombay.

2.3 *Preparation of the enzyme*

Wheat bran Czapek Dox medium containing (%): wheat bran 5, $NaNO_3$—0·2, KH_2PO_4—0·1, KCl—0·05 and $MgSO_4.7H_2O$—0·05 in water was used. Fructose solution and medium were adjusted to pH 6·2 and mixed after sterilization. Inoculation was performed by the addition of spore suspension of organism at 5% level. Incubation was carried out at 26 ± 2° under static conditions, for 5 days unless otherwise mentioned. After incubation, contents of the flasks were filtered through previously weighed Whatman No. 41 filter-paper discs and dry mat weights were recorded by drying the filter-papers with mats at 60° for 20 hours. Culture filtrates were centrifuged at 5000 × g for 15 min. The clear supernatant was used as the source of enzyme.

2.4 *Assay of protease activities*

Protease activities were assayed at pH 7·0 and 9·0 for neutral and alkaline protease respectively by the method of Kunitz[6] as described earlier by the authors[7].

2.5 *Protein determination*

Proteins in TCA (Trichloroacetic acid) precipitates of culture filtrates were measured by the method of Lowry *et al*[8], using bovine serum albumin as standard.

2.6 *Protease units and specific activity*

One unit of protease activity is defined as that amount of enzyme which liberates one mg equivalent of peptide fragments under the assay conditions. Specific activity is the units of protease activities per mg of protein.

2.7 *Determination of intracellular protease activity*

Mycelial mats obtained after filtration of culture medium were washed thoroughly with 0·9% NaCl, ground with acid–washed sand (3 g/g wet weight of the mycelia) at 0–4° and extracted with five volumes of 0·02 M sodium phosphate buffer, pH 7·0. The mixture was held at 0–4° for two hours and was then centrifuged at 12,000 × g for 20 min. The clarified supernatant was used for determination of intracellular activity. The pellet obtained was suspended in buffer and assayed for residual (cell debris associated) protease activities.

2.8 *Estimation of fructose*

Fructose in culture filtrates was estimated by the resorcinol-thiourea method of Roe *et al*[9].

3. Results

3.1 *Effect of fructose concentration*

When fructose was added at different concentrations in wheat bran-Czapek Dox medium it was observed (Table I) that with increase in concentration of fructose there

Table I

Effect of fructose concentration on the protease production by *A. alternata* (Fr.) Keissl

Concentration of fructose %	Dry mat weight mg/ml	Final pH	Enzyme units/ml	
			Neutral protease (pH 7·0)	Alkaline protease (pH 9·0)
0	4·5	6·2	0·90	1·09
0·1	4·8	6·2	1·27	1·45
0·5	4·9	6·2	2·54	2·72
1	4·7	6·2	4·36	3·63
2	4·9	6·2	6·40	5·09
3	5·0	6·2	7·21	7·60
4	5·1	6·2	6·40	5·67
5	5·0	6·2	6·40	6·40

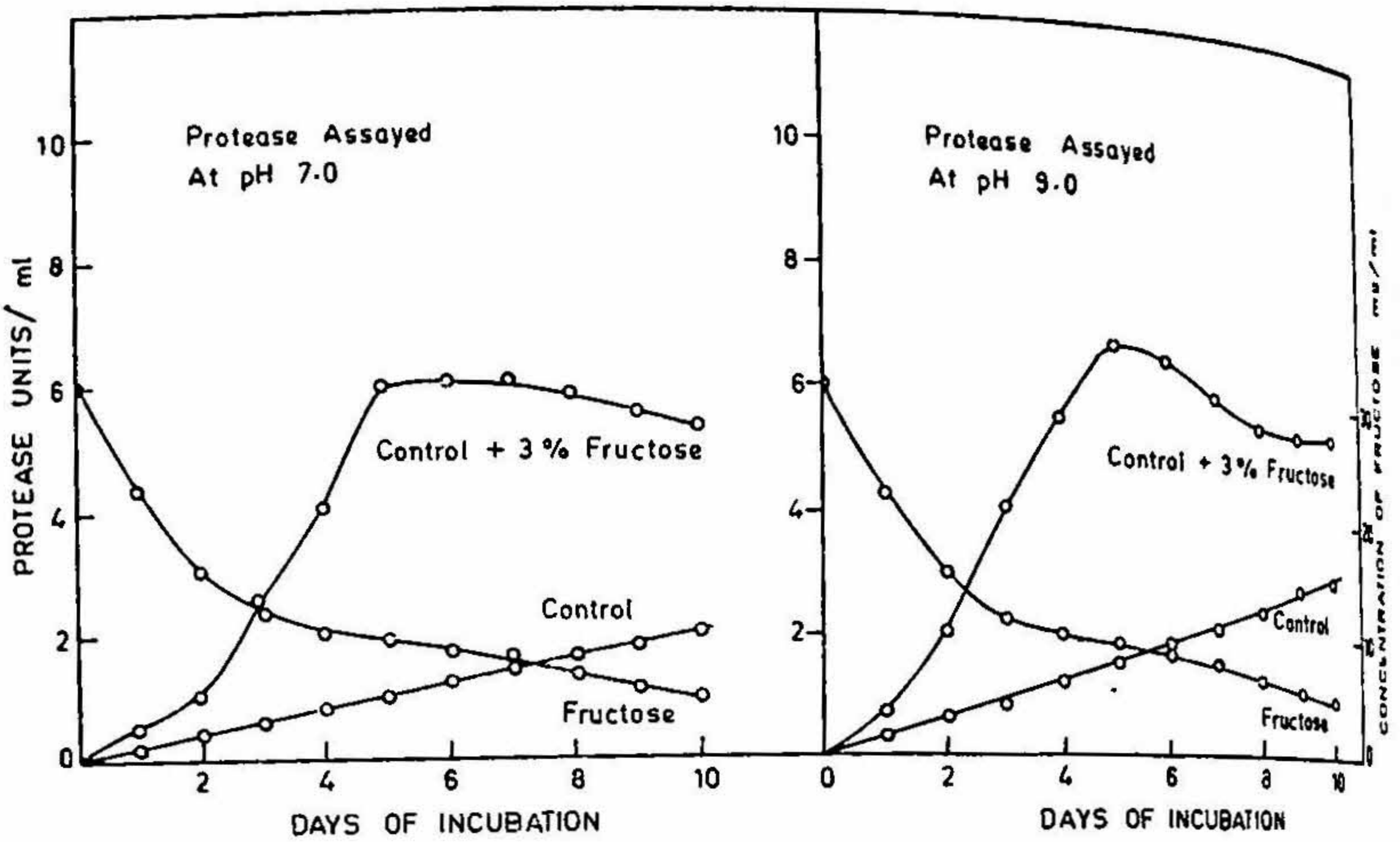

FIG. 1. Effect of incubation time on protease production by *A. alternata* (Fr.) Keissl in the presence of 3% fructose.

was a progressive increase in enzyme production, up to 3% fructose concentration. After this no further increase in stimulation of protease production was noticed. Addition of fructose in the nutrient medium did not support growth of the organism and there was no change in final pH.

3.2 *Effect of incubation time*

Nutrient medium was supplemented with fructose at 3% level before inoculation and enzymes were harvested after incubation periods up to 10 days. With increase in incubation time concentration of fructose in nutrient medium decreased (fig. 1). This is obviously due to utilization of fructose by the organism. It was also seen that stimulation of protease production by fructose, in general, increased up to 5 days of incubation after which there was no further increase in stimulation. Protease production in the absence of fructose continued to increase up to 10 days of incubation.

3.3 *Effect of addition of fructose to the actively growing culture*

Fructose was added after different incubation periods to the actively growing cultures for example at 0, 24, 48, 72 and 96 hours of incubation and enzymes were harvested after 120 hours of total incubation. Results from Table II indicate that 'stimulation' of protease production by fructose is seen within 24 hours of fructose addition. A flask containing no fructose was also run simultaneously and was observed as control

Table II

Effect of addition of fructose to the actively growing culture of *A. alternata* (Fr.) Keissl after different incubation periods

Time of addition of fructose	Dry mat weight mg/ml	Enzyme units/ml	
		Neutral protease (pH 7·0)	Alkaline protease (pH 9·0)
No addition	4·52	0·90	1·09
0 hours	4·6	5·09	4·90
24 „	5·2	5·26	4·36
48 „	5·0	5·44	4·53
72 „	4·95	5·26	5·44
96 „	4·8	5·44	5·26

3.4 *Time course study of production of proteases when fructose was added after different incubation periods*

When fructose was added after different incubation periods it was observed that stimulation of proteases increases for 5 days after addition of fructose irrespective of the period of preincubation. When the culture in which fructose was exhausted was resupplemented with fructose the stimulatory effect was again restored and continued up to 5 more days. Results are shown in fig. 2.

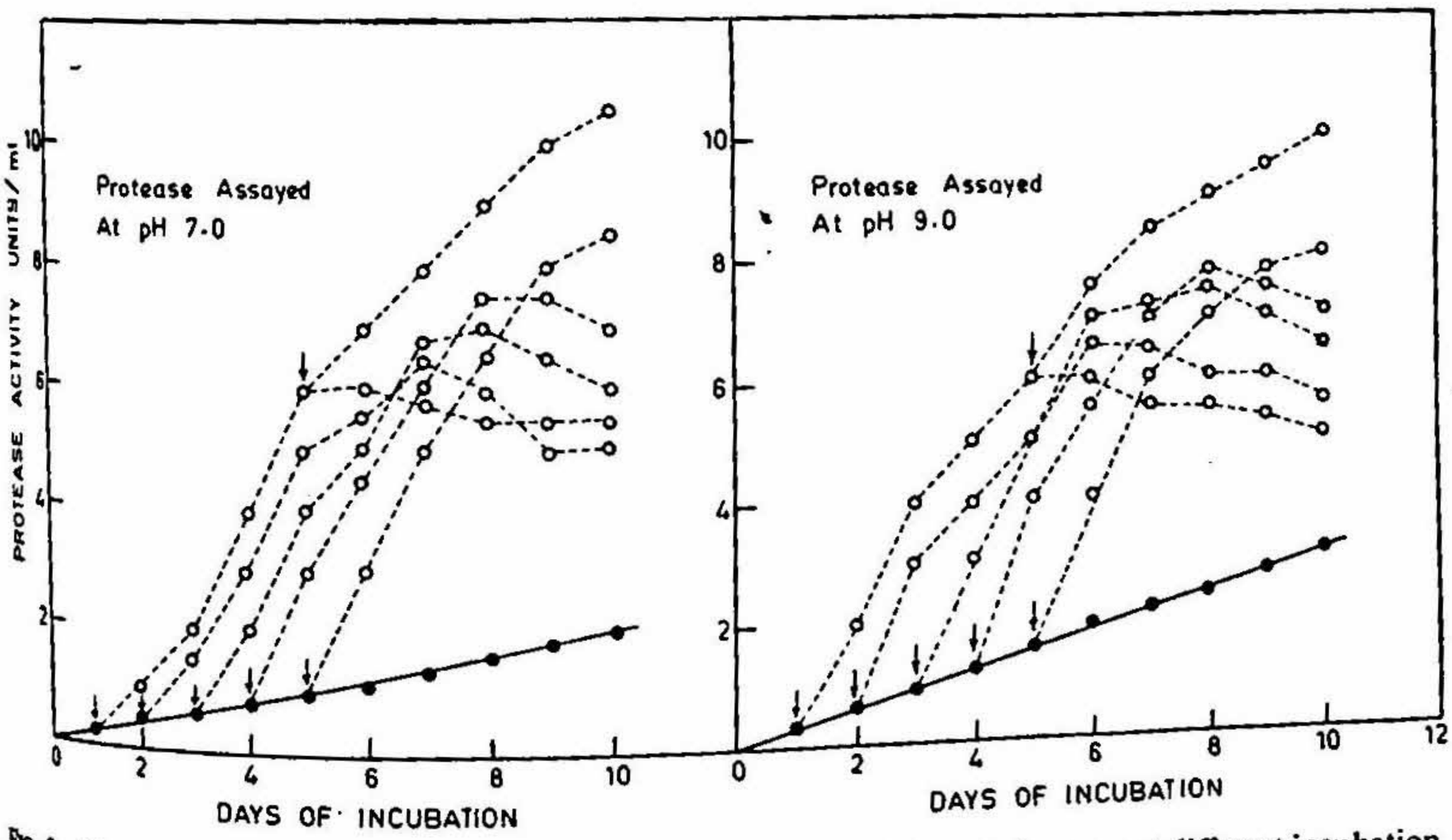

Fig. 2. Time course study of production of protease after the addition of fructose at different incubation periods. (○) + 3% fructose (●) no addition. Arrows indicate the time of addition of fructose.

3.5 *Effect of fructose, intracellular material and cell debris fraction on protease activity*

Addition of fructose (0·1–3%), intracellular material (0·047 mg protein) and cell debris fraction (0·16 mg protein) in assay mixture of proteases produced in flask containing no fructose did not activate the enzyme. This indicated that fructose did not give rise to any activator of protease in the cell.

3.6 *Effect of fructose on secretion of enzyme into culture medium*

When protease activities in intracellular, cell debris fraction and culture filtrate were measured, it was observed that total units of the intracellular and cell debris fraction were always very low as compared to extracellular activities, irrespective of whether fructose was introduced into the medium or not (Table III).

3.7 *Effect of rifampicin and chloromycetin*

To examine if fructose acts as an inducer of protease synthesis, rifampicin and chloromycetin were included in the culture media. These substances at 10^{-4} M level were found to lead to decreased production of proteases (Table IV) without significant inhibition of growth. It was also seen that the flasks containing fructose plus rifampicin and fructose plus chloromycetin gave lower protease production than the flask containing fructose alone. This indicated that fructose probably acts at the enzyme protein synthesis level.

When rifampicin and chloromycetin were added to the actively growing cultures (72 and 96 hours of incubation) it was seen that protease secretion was not affected for 24 hours after addition of the inhibitors. Inhibitory effect of these compounds manifested itself after an initial lag period of 24 hours. Protease activity of the intracellular and cell debris fraction did not appear to have been affected by either rifampicin or chloromycetin (fig. 3).

Table III

Effect of fructose on the secretion of proteases by *A. alternata* (Fr.) Keissl measured after 5 days incubation

Addition to medium	Total protease units					
	Extracellular		Intracellular		Cell debris	
	Neutral	Alkaline	Neutral	Alkaline	Neutral	Alkaline
0	28	30·78	9·8	11·24	16·64	22·64
3% fructose	238	273	13·6	13·6	13·31	23·06

Table IV

Effect of rifampicin and chloromycetin on protease formation by *A. alternata* (Fr.) Keissl

Addition to medium	Dry mat weight mg/ml	Enzyme units/ml	
		Neutral protease (pH 7·0)	Alkaline protease (pH 9·0)
0	4·6	0·81	1·09
3% fructose	4·5	6·80	6·35
10^{-4} rifampicin	4·2	0·45	0·54
3% fructose + 10^{-4} M rifampicin	4·36	1·81	1·63
10^{-4} M chloromycetin	4·36	0·54	0·09
3% fructose + 10^{-4} M chloromycetin	4·10	1·81	1·63

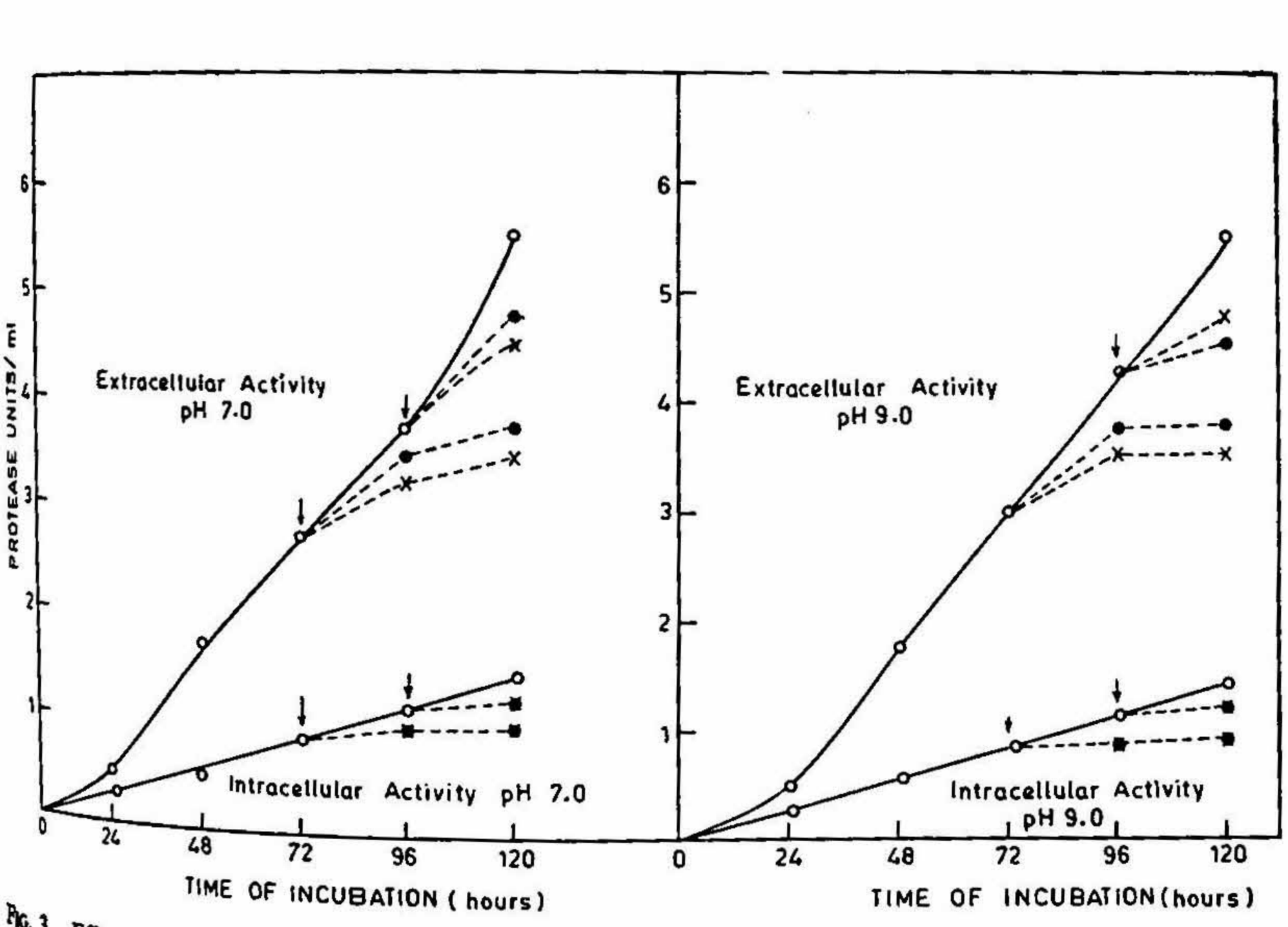

Fig. 3. Effect of addition of inhibitors on extracellular protease secretion and intracellular protease activity of *A. alternata* (Fr.) Keissl.

4. Discussion

Experiments presented in this paper indicate that stimulation of protease production of *A. alternata* (Fr.) Keissl by fructose is apparently due to its effect on biosynthesis of enzyme proteins.

Fructose did not activate the proteases *in vitro*. Intracellular and cell debris fraction from fructose containing flask when added to the assay mixture also did not stimulate the enzyme activities. This indicated that fructose did not give rise to any activator of the enzyme in the fungal cell. Since fructose did not appear to change the level of enzymes in the intracellular fraction and cell debris, a possibility that fructose might affect the secretory properties of cell membrane and stimulate the secretion of proteases into extracellular fluid appears unlikely. If fructose had affected the secretion of the enzyme and not its production, the level of enzyme in the intracellular and cell debris fraction of fructose-treated cells would have been lower than that of normal cells. Studies of Broomke and Hammel[10] with *Serratia marcescens* indicate that lower production of proteases in presence of glycerol was not on account of the accumulation of enzyme in the cell due to change in the secretory properties of the cell membrane but was the result of repression of proteases by glycerol.

The observation that increase of protease production by fructose is inhibited by rifampicin indicates that fructose might be implicated in the biosynthesis of proteases. It is known that rifampicin primarily acts at the level of transcription and inhibits the synthesis of *m*RNA[11]. Results similar to those reported by us have been noted by Broomke and Hammel[10], who found that gelatin in the medium induced the production of proteases by *Serratia marcescens*, which were also inhibited by rifampicin.

It has been suggested that extracellular enzymes produced by several microorganisms are more susceptible to the action of inhibitors of protein synthesis although total protein synthesis may not be affected[12–15]. Both *et al*[12] have suggested that there are two classes of ribosomes ; one type located at the periphery associated with cytoplasmic membrane and mainly engaged in the synthesis of enzymes which are secreted out. The process of protein synthesis which occurs at cytoplasmic membrane gets more readily affected by the inhibitor of protein synthesis. The other type of ribosomes are distributed throughout the cytoplasm and are associated with the synthesis of other cellular proteins which appears to be less sensitive to inhibitor action. Canceda and Schlesinger[16] have provided direct evidence that the periplasmic alkaline phosphatase of *Escherichia coli* is synthesized on polysomes associated with the membrane.

Addition of rifampicin and chloromycetin at the start completely inhibited the protease production. If rifampicin and chloromycetin are added to the actively protease secreting cells, protease production continues for some time. This suggests that preformed *m*RNA pool continues to synthesize proteases until it is exhausted. Micro-

organisms are known to maintain a pool of *m*RNA resulting in a possible imbalance of transcription over degradation of messengers[12,18,19]. The secretion of extracellular enzymes in the absence of messenger synthesis has been observed in *Pseudomonas kmoignei* and genus *Bacillus* and appears to be a key aspect of exoenzyme secretion in prokaryotes[12,17–19].

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