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PROTEOLYTIC ACTIVITY
IN PLANT TISSUE AND CELL SUSPENSION CULTURE

by

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ABSTRACT

Proteolytic enzymes are common in plants but are usually specific to endogenous protein. Plant proteases with specificities applicable to the food industry include papain, ficin and bromelain. Other plants have been used in traditional methods of food preparation for their proteolytic action on food components. The following species were investigated for propagation in tissue culture: Carica papaya, Ficus carica, Cynara cardunculus, Galium verum, Cirsium arvense, Dieffenbachia amoena, D. picta, and Ananas comosus. Tissues of the first five of these demonstrated proteolytic activity by clearing of milk turbidity in agar medium.

Commercial papain and ficin preparations are currently obtained from latex of immature papaya and fig fruit, respectively. This investigation was conducted, in part, to determine the feasibility of producing these two enzymes by the in vitro cell culture technique. Standard methods of aseptic seed germination and leaf tissue excision were employed for callus initiation. Cell suspension cultures derived from callus were maintained in B5 medium at 28 °C in darkness. Proteolytic activity was determined by a modification of the Food Chemicals Codex method for papain, and protein content was determined by Bradford's dye-binding method.

Production of protein and protease varied among cell cultures, but could be influenced by changes to some nutritional factors. Fig cells were grown in medium supplemented with single amino acids in the presence of either nitrate or ammonia as a source of inorganic nitrogen. All nitrate-based media produced higher yields of cell dry weight than ammonia-based media. Glutamic and aspartic acids were most stimulatory

growth, protein accumulation and protease activity of fig cells. Skimmed milk, added at 3% (v/v), was a highly effective growth stimulant, and also resulted in higher protein and protease levels than the amino acids. Fresh casein and whey, added individually, produced similar results to skimmed milk. Citric acid, added at the level found in the 3% milk supplement, also caused stimulation of fig cell growth, protein synthesis and protease activity not significantly different from skimmed milk. It appears that nitrogen accumulation and reduction in fig cells may have been limited by an energy requirement which could be satisfied with the addition of citric acid or milk whey to the basal medium.

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I. INTRODUCTION

Many of the substances used in the pharmaceutical, food, flavour and perfume industries originate from plants, and although the trend over the last fifty years has been towards chemical synthesis, plants still remain an important source of many of these compounds, for a number of reasons.

- (1) The compounds are difficult and/or costly to synthesize.
- (2) Complex mixtures, eg. rose oil, cannot be constituted successfully by man.
- (3) Isolation from a natural source circumvents many of the regulations which must be satisfied before a chemically synthetic compound can be used commercially as a food or drink additive.
- (4) Chemical synthesis may, depending on the compound, result in a mixture of isomers which cannot be separated on a commercial scale. This is of obvious importance when the major desirable property resides with one isomer.

The majority of commercially useful substances originate from plants grown in tropical and sub-tropical regions of the world and the availability and cost of these materials is frequently affected by political and economic considerations in the countries of origin.

These comments from a publication by Yeoman et al. (1980) concisely summarize the reasons for and justification of research in plant tissue and cell culture. We have a dependence on plants to provide us with much more than energy and nutrients. Most food systems are complex, including such minor components as pigments, gums, enzymes, essential oils

and other flavour compounds, most of which are derived from plants.

With the goal of self-sufficiency, plant breeding programs are aimed at adaptation of plants to temperate climates or greenhouse growth (Bozzini, 1980). The time required for development of new cultivars and hybrids was drastically reduced by tissue and organ culture. The groundwork for this approach had already been laid by researchers interested in rapid methods of plant propagation, hybridization, elimination of or resistance to pathogens (Reinert & Bajaj, 1977; Ingram & Helgeson, 1980). Several important compendia, containing articles dealing with these issues, have been edited by Barz et al. (1977), Reinert and Bajaj (1977), Thorpe (1978), Sharp et al. (1979) and Sala et al. (1980).

From plant development via tissue culture, the next logical step is to circumvent the traditional plant form and proceed directly to isolate the compounds of interest from plant cells grown in vitro (Klein, 1960).

Commercial use of enzymes is widespread: the food industry is only one among many which also include photochemicals, tanning, cosmetics, pharmaceuticals, surgery, biochemical research and waste treatment. As a result, research and development in the enzyme field has been largely directed to improvements in cost or efficiency of enzyme processes already in use, rather than development of new enzyme sources or processes. In the food and beverage industry, approximately 12% of annual sales of enzymes are proteases derived from plants, the remainder being microbial in origin.

Plant enzymes may be recovered from by-products of plants harvested for other reasons. The great importance of enzymes to some food industries underlines the need for research into alternate sources or methods of production. There is a plethora of microbial enzymes on the market, even a selection to perform any one function. Most, if not all, of

these are synthesized by selected strains of micro-organisms grown in large fermentation vessels under controlled conditions of temperature, aeration, acidity, nutrition and agitation. By contrast, plant enzyme production appears rather primitive, having progressed little beyond primary extraction, drying and improvement of storage stability (Ortiz et al., 1980).

Plant tissue and cell culture hold great promise with respect to industrial production of plant products. The art of plant tissue culture began about thirty years ago, and has since progressed to a young science, with respect to nutritional and biochemical aspects. A knowledge of the physiology and cell metabolic activity is necessary for each species and each plant product of interest, similar to, but more complex than, harnessing micro-organisms.

Useful compounds of plant origin generally fall into one of two classes, primary or secondary metabolites. Primary metabolites include precursors, intermediates and endproducts of metabolism in actively-growing cells. These would be more correctly visualized as intracellular pools. Such materials are likely to be subject to certain steady-state levels, so that an increase in recovery of a certain compound could only be achieved by means of an increased cell harvest. Improvements in cell growth would then lead to increased total productivity.

The pharmaceutical industries have contributed most to our present knowledge of plant secondary metabolism. Many drugs and cosmetics are derived from rare plants, or synthesized by common plants in such small quantities that large harvests and complex extraction schemes are required. Plant tissues and cells in culture have often been found to synthesize the desired materials, albeit generally in very small amounts. Because

secondary metabolites such as alkaloids are accumulated, especially in mature and even senescent tissue, their presence in plant tissue cultures provides impetus for improvements in methodology.

Aharonowitz and Demain (1980) argued against the distinction between primary and secondary metabolites, pointing out the possibility of multiple functions and concurrent production. All plant metabolism, they state, is subject to certain regulatory mechanisms. An understanding of regulatory points in metabolic pathways may be used to influence synthesis or catabolism of any compounds. The recent CRC (Chemical Rubber Co.) publication, Plant Tissue Culture as a Source of Biochemicals, edited by J. Staba (1980) provides ample evidence for interest and potential in this field. Table I lists many plant products identified in cultures: asterisked items have been produced in plant cultures in quantities at least equal to the parent plants (Zenk, 1978).

Table I Products detected in plant cultures.

(*) compounds produced in quantities at least equal to intact plants (dry weight basis)

References: Campbell, et al. (1965); Heinstein & El-Shagi (1981); Khanna & Staba (1968); Misawa (1977); Nickell (1980); Tabata, et al. (1978); Townsley (1974); Turnbull, et al. (1980); Zenk (1978).

<u>Class</u>	<u>Examples</u>
alkaloids	ajmalicine*, atropine, caffeine*, codeine, glycoalkaloids, indole alkaloids, morphine, nicotine, serpentine*, tropane alkaloids, vindoline
antileukemic/antitumor agents	camptothecin, elephantin, maytansine, harringtonene, lolamarine, vincristine
antimicrobial agents	plumbagin, and unidentified compounds in cultures of poplar, avocado, lettuce, cauliflower and marijuana
benzo-compounds	coumarin, gentisic acid, tocopherol, ubiquinone*, vanillic acid
carbohydrates	agar, sugars, polysaccharides, starch, lignin
cardiac glycosides	cultures of <u>Digitalis</u>
enzymes	amylases, catalase, dehydrogenases, invertase, kinases, myrosinase, phosphatase, proteases, ribonuclease
ethylene	cultures of mung bean, soybean, rose, flax, wheat, rice
foods; flavours; sweeteners	cultures of carrot, grape, tomato; aroma/flavours in cultures of onion, licorice, cocoa, coffee; miralin, stevioside
fragrances/perfumes	geranial, citronellol
furano-compounds	visnagin*, rutamarin
lipids/oils	mint
miscellaneous medicinals	anthraquinones*, plasmin inhibitor, ginsengoside*, L-dopa*
organic acids	ascorbic, chlorogenic, cinnamic, citric, fumaric, oxalic, shikimic, vanillic
phenolics	rosmarinic acid*, hydrangenol, putrescine (under study)
pigments	anthocyanins, betanin, carotenoids, chlorophyll, flavonoids, gossypol
steroids, saponins	campesterol, cholesterol, diosgenin*, lanosterol, sitosterol, squalene
vitamins	ascorbic acid, thiamine, vitamin K

II. LITERATURE REVIEW

1. Plant proteases: Description and uses

Proteolytic enzymes find uses in the following food industry sectors: baking, brewing, protein hydrolysate production, cheese-making and meat processing. There is also some interest in their use in waste treatment or reduction, such as hydrolysis of scleroproteins (Jones & Mercier, 1974; Yamamoto, 1975). Criteria for enzyme selection for a particular purpose include specificity, heat-stability, pH optimum and the possible presence of inhibitors in the enzyme preparation or the intended substrate.

The major plant proteases currently employed are papain, bromelain and ficin, estimated to total over \$16 million in international sales in 1980 (Wolnak, 1980). Papain is in use in many food and non-food industries, described by Jones and Mercier (1974). They pointed out the instability of crude enzyme preparations (dried latex) and the necessity for refinement and low-temperature storage. Appropriate facilities are generally confined to large enzyme companies located in North America, Europe or Japan, while the crude papain is purchased mainly from tropical nations. Ortiz et al. (1980) investigated the storage and drying characteristics of papaya latex. They reported an optimum drying temperature of 50-55°C. Proteolytic activity declined in the presence of sodium chloride while addition of EDTA to fresh latex had a preservative effect on activity.

Table II summarizes properties of the three major plant proteases, with most information available on papain. All three enzymes are monomers containing at least one cysteine residue which must be in a reduced form to be enzymatically active. Basically, catalysis depends on the formation of

Table II Characteristics of the three major plant proteases currently used in the food industry.

Data for this table came from the following references:

- | | | |
|------------------------------|--------------------|--------------------------------|
| (1) Bergmeyer, 1974 | (2) Murachi, 1970 | (3) Liener & Friedensen, 1970 |
| (4) Arnon, 1970 | (5) Yamamoto, 1975 | (6) Gould, 1975 |
| (7) Englund, et al., 1968 | (8) Tang, 1974 | (9) Kunimitsu & Yasunobu, 1970 |
| (10) Sgarbieri, et al., 1964 | | |

Similarities in primary structure near the reactive cysteine*

papain.....Pro-Val-Lys-Asn-Gln-Gly-Ser-Cys-Gly-Ser-Cys*-Trp

ficin.....Pro-Ile-Arg-Gln-Gln-Gly-Gln-Cys-Gly-Ser-Cys*-Trp

bromelain....Ser-Val-Lys-Asn-Gln-Asn-Pro-Cys-Gly-Ala-Cys*-Trp

property	papain	ficin	bromelain
source	latex of papaya fruit	latex of fig fruit	fruit or stem fluid of pineapple
molecular weight	21,000 (5,6) 23,000 (4)	24-27,000 (3) 25,500 (7)	33,000 (1,2)
terminal residues	N isoleucine C asparagine	leucine alanine	valine glycine
substrates	proteins, peptides, esters	proteins, peptides, esters	proteins, peptides, esters
specificity	basic carbonyl residues: Arg, Lys, Phe	Phe or Tyr carbonyl group	basic or aromatic carbonyl residues: Arg, Phe, Tyr
temperature	most active @ 50-60 °C, stable 30 min, 70 °C (5)	most active @ 50-60 °C	most active @ 50-60 °C
pH stability	optimum pH 5-7; stable pH 3-11(5), 3-9 (6)	optimum pH 6-8; stable pH 3.5-9 (5)	optimum pH 6-8, except gela- tin (optimum pH 5)
pI	8.75 (4)	9.0 (3)	9.55 (2)
inhibitors	heavy metals, oxidants, sorbic acid (5) isothiocyanates (8)	heavy metals, oxidants, sorbic acid (5)	heavy metals, oxidants, sorbic acid (5)
active site	cleft involving Cys ₂₅ , Asp ₁₅₈ and His ₁₅₉ (4)	cleft involving cysteine (3)	cleft involving cysteine and histidine (2)
associated proteases	chymopapain A & B (4,5,9) lysozyme	up to 10 active components (3, 10)	5 isozymes (2)
notes	synthetic activity in plastein reactions (5)		glycoprotein: 1.5-2.5% carb- ohydrate, bonded to Asn

an intermediate complex of the substrate acyl moiety with the active cysteine residue. This has been elaborated upon via several avenues of exploration as reviewed by Polgar (1977) with respect to papain. In this case, the enzyme cleft is capable of binding seven amino acid residues. One of these is the discriminating residue, second in the N-terminal direction to the point of cleavage (Fig. 1). The imidazole group of His₁₅₉ is probably coupled with the thiol of Cys₂₅ forming the active nucleophile for substrate attack. The linear separation of these amino acids is overcome by peptide folding to produce the catalytic cleft, possibly spanned by the discriminatory substrate residue. Brocklehurst and Kierstan (1973) proposed a zymogen-like mechanism for papain. They gave evidence suggesting that a thiol-disulfide interchange effects the conversion of inactive propapain to the active enzyme configuration. The basic scheme for this transition is given in Figure 2.

Less is known of the proteolytic action of ficin, but recent investigations indicate a mechanism very similar to that of papain. Two sulfhydryl groups have been demonstrated in denatured ficin, but only one in active ficin (Englund et al., 1968). Papain contains seven cysteine residues, only one of which is a reactive sulfhydryl, the other six being involved in disulfide bonds. Similarly, ficin appears to have three disulfide bonds, since the total half-cystine value is eight. Reports in the literature indicate that ficin preparations are very heterogeneous (Kramer & Whitaker, 1964; Sgarbieri et al., 1964). For this reason, precise activity of commercial preparations in complex systems cannot readily be predicted. Englund and co-workers (1968) demonstrated that the multiplicity of active fractions could be attributed to autolytic action which had not interfered with proteolytic capacity of the enzyme.

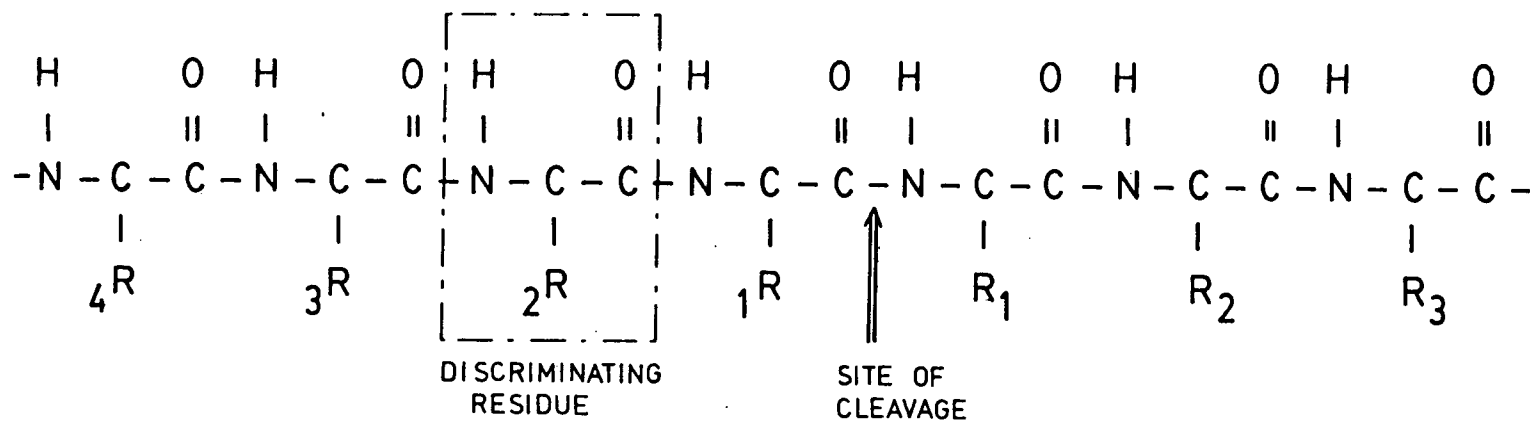


Figure 1. Substrate fragment accommodated by the papain active site. Seven amino acid residues are held in the enzyme cleft. The second residue in the N-terminal direction from the point of cleavage, the "discrimination residue", is preferentially hydrophobic.
From L. Polgar, 1977, J. Biochem. 8: 171-176.

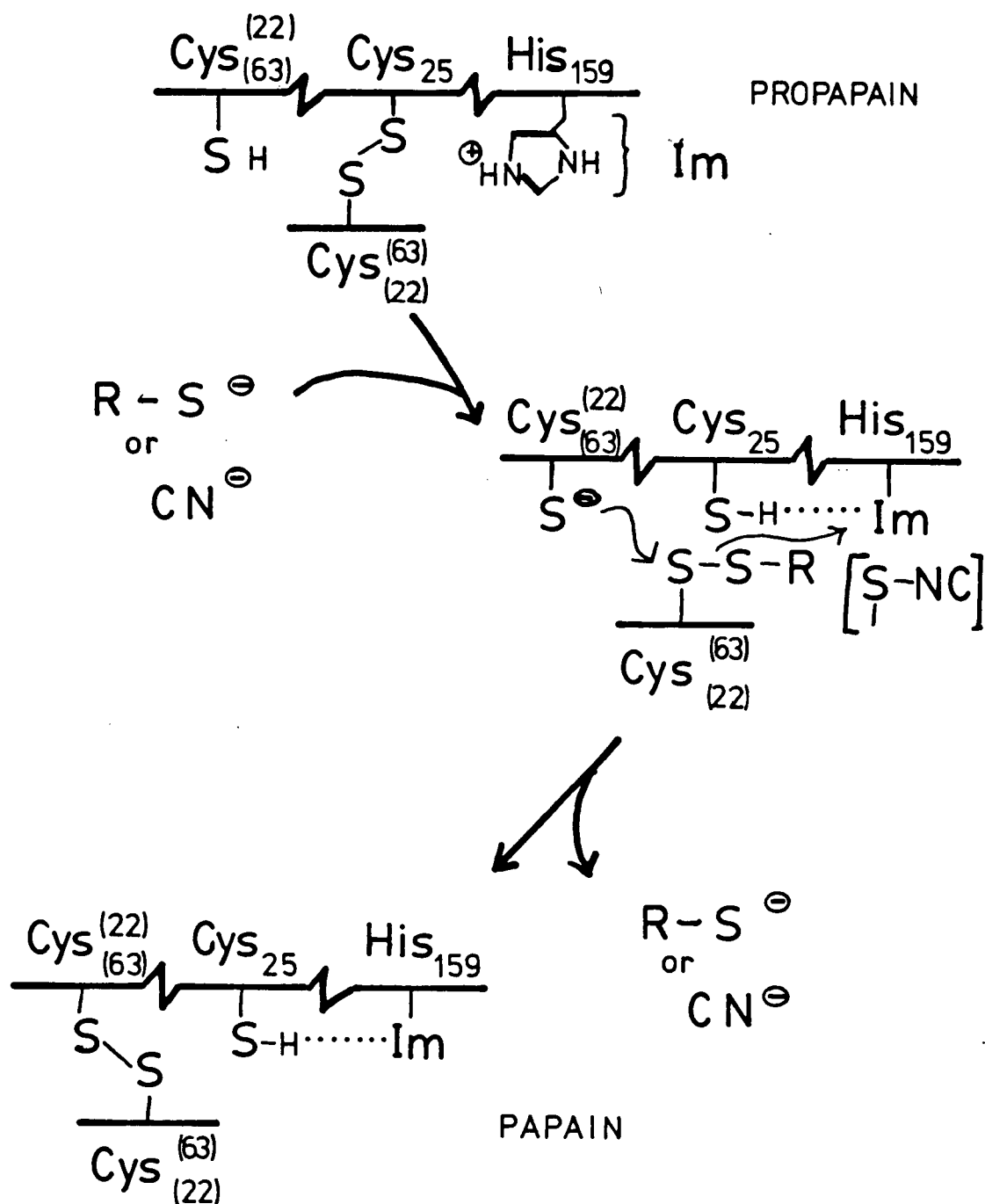


Figure 2. Transition of propapain to papain: thiol-disulfide interchange as the method of zymogen activation.

From K. Brocklehurst & M.P.J. Kierstan, 1973
Nature, New Biology 242:167-170

Most current technical information on the bromelains is outlined in Table II. Although stem and fruit bromelains are also sulfhydryl proteinases, they are glycoproteins and differ from ficin and papain in other respects. Esterase and amidase activities of the three enzymes indicate a fundamental difference: both papain and ficin show approximately equal affinity for corresponding synthetic ester and amide substrates while stem bromelain has a k_{cat} for BAEE (α -N-benzoyl-L-arginine ethyl ester) 140 times as large as the k_{cat} for BAA (α -N-benzoyl-L-arginine amide), (Murachi, 1970). Stem and fruit bromelains have half-cystine values reported between 5 and 11, with only one reactive cysteine residue.

Meat processing is the major application of plant proteases. Polynesians and Hawaiians have traditionally treated meats with papaya or pineapple, presumably for the improved texture, though flavour improvement is no doubt also important. Meat was rubbed, or even boiled, with the raw fruit or it was marinated by storing wrapped in papaya leaves. The meat industry has applied and elaborated on these ancient recipes for meat tenderization. Tenderizers are now used immediately pre- or post-slaughter and upon preparation for cooking or canning meat and fish products. The North American product retailed under the name of ProTen, no longer available, was beef tenderized by antemortem injection of papain. This has been considered a great breakthrough by the meat industry in their quest for an inexpensive and thorough means of meeting the consumers' demand for tender, yet lean, meat (Goeser, 1961). Muscle fibre proteins and collagen, including capillary walls, are hydrolyzed to some extent in vivo, and further degraded upon cooking (Kang & Warner, 1974). Organ tissues (kidneys, liver, heart, etc.) receive higher doses of the enzyme unless slaughter rapidly follows injection. Orsi and Major (1973) developed an assay procedure for routine

use in the Hungarian meat industry. By this method, activity of all three plant proteases (ficin, papain and bromelain) was found to correlate well with tenderness ratings of a panel of judges.

Plant proteases have also been investigated for potential application in the dairy industry, primarily for rennet substitution in cheese manufacture (Balls & Hoover, 1937; Cooke & Caygill, 1974). Scott (1973) and Sardinas (1976) have both listed a number of plant sources of coagulants, among them pumpkin, cardoon, sundew and the three already discussed. Scott also pointed out the two distinct functions the enzyme must perform: coagulation of the milk, allowing expression of whey but retention of other constituents, and curd hydrolysis for further microbial or enzymic action in aging, to produce characteristic texture, flavour and aroma. Ficin and papain preparations used to make cheese are too highly proteolytic, according to Scott and others (Sardinas, 1972; Cooke & Caygill, 1974; Kosikowski, 1977). This results in lower yields due to whey expression by the firm curd as well as losses due to casein hydrolysis. Furthermore, bitter flavours may develop upon ripening. A certain degree of bitterness is sometimes desirable, though, in such cheeses as roquefort. Sardinas (1976) explained patented methods for the use of bromelain, ficin and papain: the enzymes are added to milk but, when casein has been converted to paracasein, they are inactivated (eg., by peroxide treatment); then bacterial cultures are added for ripening. Another solution to bitter peptide formation might be the inclusion, in ripening cultures, of bacteria that will metabolize these products.

The extract of cardoon is reportedly still in use in Iberia for preparation of an unripened cheese (Vieira de Sá & Barbosa, 1970b; Kosikowski, 1977). Vieira de Sá and Barbosa (1972) made Edam, Serra and

Roquefort cheeses using an extract from cardoon flowers (Cynara cardunculus). They found the maximum clotting activity at 70°C but most similar to the clotting time of animal rennet at 32°C. In sheep's milk, only one-third as much extract was required as in cow's milk for suitable curd firmness. They concluded that the cardoon extract was suitable for manufacture of soft-bodied cheeses like Serra, not as good for Roquefort due to decreased yields, and unsuitable for Edam production.

Ladies' bedstraw (Galium verum) has been extracted for use in traditional cheese-making in the Middle East. It was also in use in Cheshire, England in the late 18th and early 19th centuries (Scott, 1973). Sardinas (1976) cited a report of cheese manufacture using berries of Withania coagulans, historically used in India. In summary, it appears that there are numerous plants known to have milk-clotting properties, few of which have been investigated for practical application or for identification of the coagulating principles.

If plant proteases are to find use in this aspect of the dairy industry, it is essential to develop close controls over enzyme activity. Because of this requirement, many researchers have turned to work with immobilized enzymes and "cold-renneting" techniques. To date, these methods have not produced an effective means of replacing rennet in the manufacture of traditional cheeses. Microbial rennets and pepsin are commonly used, but do not generally equal the rennet-coagulated cheeses in sensory properties (Sardinas, 1972; Sternberg, 1976).

2. Plant tissue and cell culture

Plant tissue cultures are commonly derived from excised leaf, fruit, seedling or bud tissue. These tissues are incubated aseptically on a

nutrient agar medium, several of which have been developed for general use or special purposes (Murashige & Skoog, 1962; White, 1943; Gamborg et al., 1968). Under appropriate conditions callus tissue, consisting of masses of undifferentiated plant cells, forms. In a developing callus, cell division is rapid until the cell mass begins to restrict nutrient uptake. To encourage cell proliferation, the callus must be dissected and transferred often (every 10-40 days, depending on the tissue). Plant cell suspension cultures can sometimes be established from callus by dispersion of the callus in liquid medium with appropriate agitation.

Success or failure of plant tissue and cell suspension cultures depends upon both growth conditions (media, temperature, aeration) and genetics (species, plasmids, nucleic acid replication). There is a large body of literature on manipulation of these two factors (see Reinert & Bajaj, 1977, and Thorpe, 1978, for example). Dougall (1980) has presented a concise and very readable synopsis on the current status of nutrition research in plant tissue and cell culture. Much like the development of knowledge regarding microbial metabolism, plant cell cultures are the subject of numerous ongoing investigations: carbohydrate and nitrogen metabolism, micronutrient requirements and functions, plant hormone activities and the relationship of nutrition to differentiation and senescence. New cultures are initiated in media proven successful for other species and, if adequate, are then modified by supplementation or omission of nutrients. Such work requires dedication and the investment of many years of research, the contributions of O.L. Gamborg (1975) and H.E. Street (1977) being outstanding examples.

Plant tissue culture has been quite successful in horticultural endeavours (Boxus & Druart, 1980; Murashige, 1978). As discussed in the

introductory chapter, this has become a popular and expanding field in pharmaceutical research as well. Since the advent of axenic culture of plant tissues, there have been a few attempts to grow papaya tissue in vitro. The first report of papaya callus cultures was probably that of Medora et al. (1973), in which they gave evidence of proteolytic activity in these cultures. They have subsequently published information about medium composition and substrate specificities (Bilderback et al., 1976; Medora et al., 1979; Mell et al., 1975, 1979). Enzyme activity was first assessed using a casein substrate to which buffered extracts of mortar-ground, lyophilized tissue was added (Medora et al., 1973). Later work showed that azocasein and casein yellow were better suited for assay of crude extracts, while hemoglobin, azocoll, and hide powder azure were all inadequate. Medhi and Hogan (1976, 1979) have also propagated papaya callus and produced plantlets from embryoids. Arora and Singh (1978) found that the most effective growth hormones for papaya callus development were NAA (naphthalene acetic acid, 1.0 mg/l), kinetin (0.5 mg/l) and gibberellic (1.0 mg/l) acid. Propagation of papaya plants via tissue culture, however, required changing of hormone concentrations during plantlet development (Litz & Conover, 1978).

Apte et al. (1979) reported proteolytic activity in tissue cultures of pineapple. Callus, initiated from lateral buds, was grown in the presence of NAA (10 mg/l), casein hydrolysate (0.4 g/l) and 15% coconut milk. This group extracted the enzyme(s) by acetone fractionation of blender-homogenized tissue. Using a casein hydrolysis assay method, they found great fluctuations in activity over a 50-day period, but proteolysis was lower in calli and regenerated plantlets than in the mature plant. No one has yet reported attempts to improve protease yields or to produce

active cell suspension cultures from papaya or pineapple.

Other species investigated herein have not previously been studied as undifferentiated tissue. Litz and Conover (1977) propagated Dieffenbachia from excised lateral buds, with a minimum of callus formation. There appear to be no reports in the literature regarding in vitro propagation of fig.

The focal point of this research project was to assess proteolytic enzyme activity in cell suspension cultures derived from plants known to produce proteases. Preliminary work therefore required preparation of callus cultures from a number of plant sources: papaya, fig, pineapple, cardoon, Dieffenbachia and bedstraw. From this stage, it was necessary to develop cell suspension cultures. Successful establishment of these then led to an evaluation of protease activity. All subsequent work was aimed at stimulation of proteolytic enzyme production and activity in cell suspension cultures, with concomitant observations on growth and total protein production under the test conditions.

III. MATERIALS and METHODS

1. Callus culture of selected species

General methods and conditions: The following seven plants were used as tissue sources for callus culture: papaya, fig, cardoon, dumbcane, pineapple, bedstraw and thistle. A similar procedure of excision, sterilization, trimming and plating was followed for all tissues, with detailed methods given below.

Explants and calli were incubated in plastic basins covered with aluminum foil to maintain humidity and minimize contamination and light exposure. Incubation was at a temperature of $28 \pm 3^{\circ}\text{C}$ and in total darkness, so that cultures were exposed to light and temperature fluctuations only for brief periods, no longer than one hour, during observation and transfer. A sterile transfer cabinet of the horizontal laminar air flow type was used (Enviroco, Becton Dickinson Co., USA). Disposable 5 cm petri dishes were used throughout. Table III gives the composition of B5 medium (Gamborg et al., 1968), the medium most commonly used: MS medium (Murashige & Skoog, 1962) was used for some young tissues. Bacto-agar (Difco) was added at 0.6% (w/v) for callus tissues. Media were sterilized in an autoclave at 15 psi and 121°C for 15 min. Developing calli were assessed for protease activity by visible clearing of B5 agar media containing 3% skimmed milk, B5-M.

Carica papaya: Papaya was propagated from explants of germinated seeds as outlined in Figure 3. The seeds were aseptically removed from a market papaya and the arils peeled off each seed. The seeds were then soaked in two rinses of sterile distilled water for about 5 minutes each then blotted dry with sterile filter paper. At this point, about half the seeds so pre-

Table III

B5 Medium and Plant Hormones

From Gamborg, Miller and Ojima (1968): Exp. Cell Res. 50: 151-158

<u>Salts</u>	$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	150 mg/l
	KNO_3	2500 mg/l
	$(\text{NH}_4)_2\text{SO}_4$	134 mg/l
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	250 mg/l
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	150 mg/l
	KI	0.75 mg/l
	Fe (Sequestrene 330-Iron)	28 mg/l
<u>Micronutrients</u>		1.0 ml/l
	stock solution (in 100 ml)	
	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	1.0 g
	H_3BO_3	0.3 g
	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.3 g
	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	25 mg
	CuSO_4	25 mg
	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	25 mg
<u>Vitamins</u>		10 ml/l
	stock solution (in 100 ml)	
	nicotinic acid	10 mg
	thiamine	100 mg
	pyridoxine	10 mg
	myo-inositol	1 g
<u>Carbohydrate</u>	sucrose	20 g/l
<u>Plant Hormones</u>	IAA (indole-3-acetic acid)	1.0 mg/l
	2,4-D (dichlorophenoxyacetic acid)	1.0 mg/l
	or p-cpa (parachlorophenoxyacetic acid)	1.0 mg/l
	or 2,4,5-T (trichlorophenoxyacetic acid)	1.0 mg/l
	kinetin (6-furfurylaminopurine)	0.1 mg/l
<u>Adjuncts (optional)</u>	agar	6.0 g/l
	skimmed milk	30 ml/l
	casein hydrolysate	0.5-2.0 g/l
	yeast extract	0.5-2.0 g/l

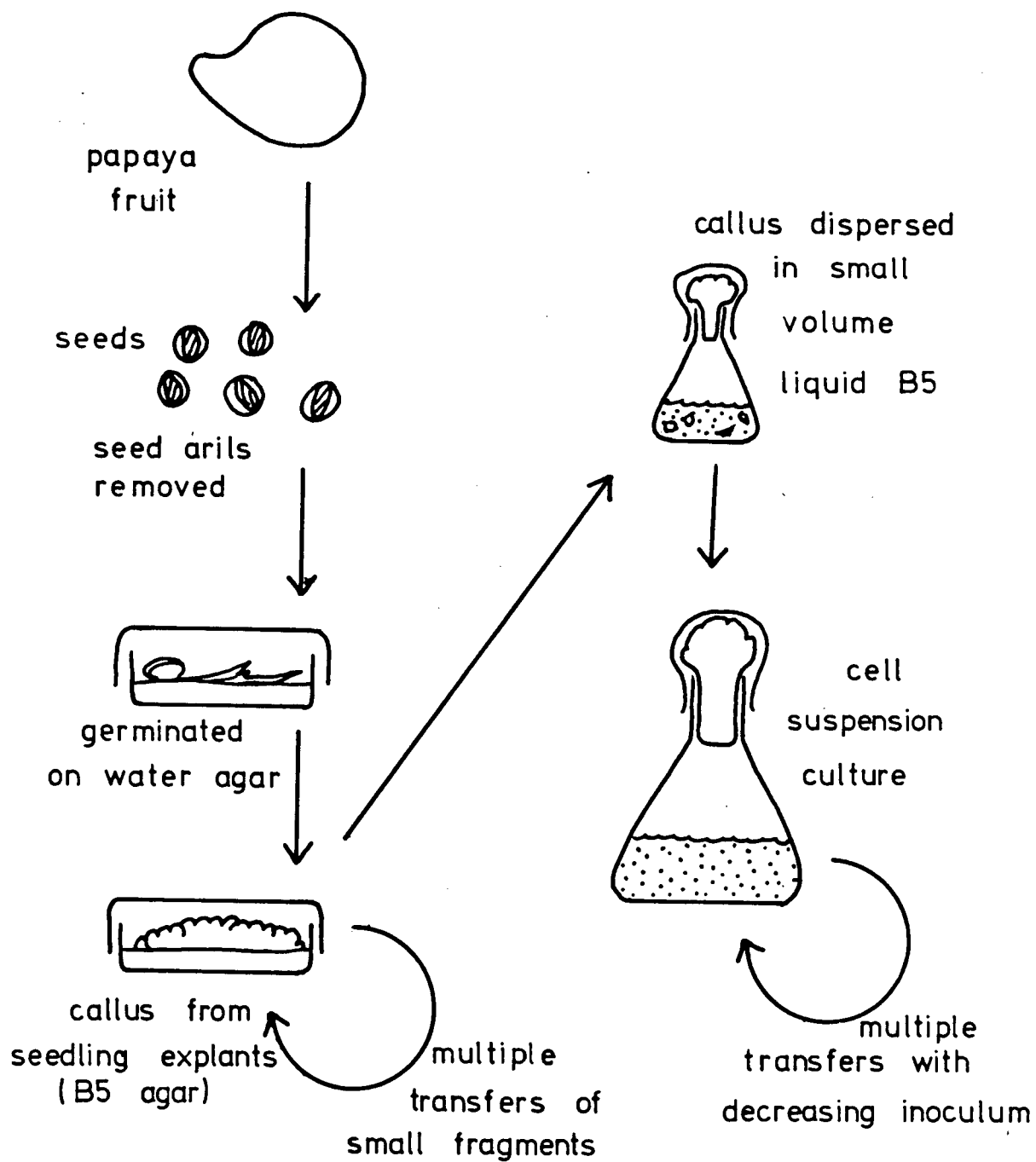


Figure 3. Preparation of papaya for tissue and cell culture. All seeds, callus and cell suspension cultures were maintained at 28 °C in darkness.

pared were mechanically damaged so as to break the tough seed coat: the other half were left intact. All seeds were placed on sterile distilled water-agar (WA) in petri dishes with a minimum surface area of 5 cm² per seed. Plates were incubated as described above until germination. About the fourth day after germination, the new seedlings were aseptically explanted and segments no longer than 2 cm transferred to nutrient media. These were based on B5 or MS and generally contained 2,4-D (dichlorophenoxyacetic acid, 1.0 mg/l), IAA (indole-3-acetic acid, 1.0 mg/l) and kinetin (6-furfurylaminopurine, 0.1 mg/l). Plates containing explants were incubated under the same conditions and observed for callus formation in 2-5 weeks. When callus tissue was approximately five times the original explant size (estimated tissue volume), it was dissected away from the parent tissue and transferred to fresh agar medium. Such passages were repeated a minimum of three times, with at least two weeks' growth each time, prior to further experiments or propagation in liquid media. The inoculum selected for transfer was always near the fringes of the callus so as to take only very young, rapidly-growing tissue.

Ficus carica: Fig tissue cultures were initiated from leaves of fig trees. The leaves were prepared for explantation by gently washing large pieces of both laminae and petioles under running water then soaking 5-10 minutes in two washes of 10% commercial bleach, followed by rinsing in three changes of sterile distilled water, 10 minutes each. Cut edges were aseptically trimmed, exposing fresh surfaces, and these pieces cut into explant fragments averaging 1 cm in length for petioles and 1-2 cm² in area for laminae. These explants, blotted dry, were transferred to B5 or MS agar plates and incubated under conditions described above. After one week, all were transferred to fresh agar then incubated again until callus was

observed. Callus tissue at least equal to the original explant in size was dissected free and transferred to fresh agar. At least three passages of young callus tissue preceded attempts at liquid culture.

Cynara cardunculus: Seeds of cardoon were a gift of Nichols Garden Nursery, Albany, Oregon, USA. The seeds were sterilized by soaking in 15% commercial bleach (2 x 10 min) and rinsing in sterile distilled water (3 x 10 min). Some were cracked, others left intact, then germinated on WA as described for papaya. Explants of seedlings and resulting callus were handled as described above.

Dieffenbachia amoena & D. picta: Leaves of two species of dumbcane were obtained from the UBC Plant Science Department greenhouses. They were prepared according to the method given above for fig. The high contamination rate, however, required much more severe sterilization protocol: 1 x 10 min and 1 x 20 min in 15% bleach, followed by 3 sterile water rinses totalling 30-40 min. Otherwise, the culture method was the same as given for fig. The auxin, 2,4-D, was replaced with 2,4,5-T (trichlorophenoxyacetic acid).

Ananas comosus: Pineapple tissue was derived from two sources, the market fruit and vegetative plant, both purchased retail. Basal leaf explants from the tops of pineapples were prepared as described for fig, using final explants less than 1 cm² in size. Cross-sections of vegetative leaves including base and leaf margins were sterilized as described for dumbcane. The latter procedure was also applied to explants from the flesh of slightly under-ripe fruit. Standard media (B5 and MS) were used, except for the use of 2,4,5-T.

Galium verum: Cultures of ladies' bedstraw originated from freshly-harvested seeds. These were sterilized according to the method given for cardoon, but with the addition of a wetting agent to the first bleach soak

(2 drops Palmolive detergent per 100 ml bleach solution). Bedstraw seeds carry dense surface dispersal appendages which would otherwise prevent wetting and effective sterilization. Explants of the germinated seedlings were 0.5-1.0 cm in length. Incubation conditions were as described above. Numerous modifications of B5 medium were made in attempts to encourage callus formation, rather than the fine root-like proliferative structures which developed. These modifications included all the hormones listed in Table III, 0.05-0.5% casein, 1-5% skimmed milk, 10% coconut milk, 0.2% yeast extract and 4 mM thiourea. Effects of these modifications were assessed by visual examination for callus initiation.

Cirsium arvense: Wild thistle (var. horridum) was collected fresh prior to excision. Leaves and stems were surface-wetted with 70% ethanol because of surface hairs, then sterilized in two 5-10 min soakings in 15% bleach and rinsed in sterile water (3 x 10 min). Trimmed explant dimensions were 0.5-2.0 cm. Standard media and incubation conditions were used.

2. Suspension culture of fig and papaya

Only fig and papaya calli proliferated rapidly enough to establish viable cultures in liquid media. B5 was the basal medium with the following hormones: kinetin (0.1 mg/l), IAA (1 mg/l) and p-cpa (p-chlorophenoxyacetic acid, 2 mg/l). Cell suspension cultures began with dispersal, in liquid medium, of large callus tissues representing at least 10% of the final volume. Flasks of more than double the requisite capacity were used so as to provide a large surface area for aeration of the medium. They were stoppered with cheesecloth-wrapped cotton and further protected with two layers of paper towelling secured about the flask necks with elastic bands. Incubation conditions were constant: $28 \pm 3^{\circ}\text{C}$, dark, 110 rpm on rotary New

Brunswick shakers. After 1-4 weeks' growth, depending on how readily the callus sloughed cells and the suspension thickened, these primary cell suspension cultures were transferred to fresh media using a large-bore pipette and an inoculum size of 10-15% of the final volume. Herein, these will be referred to as "cell suspension cultures" or "cell cultures", according to traditional terminology in the literature. A minimum of three such consecutive transfers preceded further experimentation in cell line selection, protease assay or medium composition as described below.

3. Assay methods

Biomass: Cell dry weight obtained from initial cell suspension volumes (ie., medium + inoculum) were converted to yields based on one litre. Dry weights were determined by three methods: to constant weight in a convection oven at 60 °C, in a vacuum oven at 60°C, or in a Virtis freeze-dryer (low heat, condenser temperature -60°C, less than 1 mm Hg). Cells were harvested by filtration through Miracloth (Chicopee Mills, Inc., NJ, USA) then transferred to pre-dried, pre-weighed aluminum dishes. After establishment of the relationship between results of these methods, all harvest weights were derived from lyophilized samples since these were then used for enzyme assays.

A rapid method of assessing cell growth is based on "settled cell volume" (Nickell & Maretzki, 1969), also employed by Behrend and Mateles (1975). This method was compared to cell dry weights to evaluate its validity because of the advantage that results could be obtained immediately upon harvest. Cell suspension cultures were allowed to settle about 30 min. The medium was decanted through Miracloth to trap floating cells and the thick cell suspensions poured into large graduated centrifuge tubes.

Cells collected on the filter were also added, by scraping of the filter on a flat surface. Tubes were centrifuged 3-5 min at 200 xg and the settled cell volume was read directly. The cells were then lyophilized to obtain dry weights.

Cell extraction: Three methods were compared for extraction of intracellular material to determine the most effective one for release of proteins and, in particular, active proteases. Cell disruption was accomplished by sonication (80 W, 30 & 120 sec; Braunsonic 1510), by homogenizing (1-4 min, Polytron Kinematica PCU-1, Brinkmann Instruments) and by grinding in a mortar with or without washed sand as an abrasive (45-90 sec at an average rate of 60-80 strokes/min). The last of these became the standard method for further studies. In all cases, cells were suspended in ice-chilled extraction buffer, 0.1 M phosphate at pH 6.0, and kept on ice throughout. These crude extracts were filtered through Miracloth to remove cell debris then assayed for protein content and proteolytic activity.

Protein content: Crude cell extracts were kept on ice and assayed for protein content within three hours. The dye-binding method of Bradford (1976) was used routinely due to the simplicity (one reagent, Coomassie Brilliant Blue-G, Sigma) and rapidity (10 min). This method was compared to protein determinations by three other methods. Ultraviolet light absorbance at 280 nm (Beckman DB spectrophotometer) could not be determined accurately without filtration and high-speed centrifugation of the extracts due to interference caused by cloudiness. Since this step would also pellet some of the protein, it was considered unsuitable. Nitrogen content of cells was determined by the micro-Kjeldahl method (AOAC, 1975), and quantitated by a Technicon Autoanalyzer II nitrogen analyzer. Finally, the Lowry procedure (Lowry et al., 1951) was tested. A dilution series of bovine serum albumin

(Fraction V, Sigma) was included as the standard protein for Bradford's and Lowry's methods.

Protease activity: The method of the U.S. National Research Council's Food Chemicals Codex (1966), hereafter referred to as the FCC method, was selected. This procedure was modified as follows: Hammarsten casein (BDH) substrate concentration was 0.2 g/100 ml; extraction buffer (as previously described) was used in place of activation buffer; the incubation period was extended to 2 hr, and dithiothreitol (Dtt, Sigma, 2 mM final concentration) replaced cysteine as the activator, added with EDTA (G. Frederick Smith, Ohio, USA, at 0.4 mM final concentration) immediately prior to incubation. Samples were both centrifuged (8,000 xg, 10 min, Sorvall RC-2) and filtered prior to reading the absorbance at 280 nm. Each sample evaluation consisted of four tubes; two incubated sample/substrate/activator reaction mixtures and two incubated substrate/activator mixtures to which sample was added post-incubation, along with TCA (trichloroacetic acid, Fisher).

Results of this method were compared to trials of seven others:

- (a) digestion of Hide Powder Azure (a dye-labelled collagen, Sigma) according to Savage and Thompson (1970), using 0.5 ml of extract diluted to 5.0 ml in place of beer, and extending the incubation time to 1 hr;
- (b) esterase activity by pH-dependent indication of radial diffusion according to Araki and Abe (1980), using BAEE (N-benzoyl-L-arginine ethyl ester, Sigma) as the substrate;
- (c) caseinolytic activity by the agar-diffusion method of Holmes and Ernstrom (1973);
- (d) caseinolytic activity using the Bio-Rad "Protease detection kit" (1978);

- (e) gelatin digestion from film, by the method of Glenister and Becker (1961), using 0.5 ml extract diluted to 5.0 ml instead of beer, and Kodak Panatomic-X film;
- (f) fluorescence loss of ANS (1-anilino-8-naphthalenesulfonate, Mg salt, Sigma) according to Spencer and Spencer (1974), using the casein substrate and activated (Dtt and EDTA) crude cell extracts (Aminco-Bowman spectrophoto-fluorometer; excitation and emission wavelengths, 370 and 460nm, respectively);
- (g) development of fluorescence by reaction of fluorescamine (Sigma) with TCA-soluble casein digestion products, according to the method of Chism et al. (1979), using 0.2% Hammarsten casein without sodium azide incubated with 0.025-0.5 ml crude cell extract for 1 hr prior to precipitation and reading (excitation and emission wavelengths, 390 and 475nm, respectively).

In all cases, the samples tested were filtered extracts of lyophilized cells ground in a cold mortar with ice-chilled buffer as described above. Appropriate concentrations of purified papain (African, Calbiochem) or purified ficin (Sigma) enzyme standards were used, and are referred to as "standard" throughout.

The FCC method was selected for routine protease determinations. Supernatant absorbance at 280 nm is dependent on the quantity of 10% TCA-soluble aromatic amino acids released from the casein substrate during the incubation period. Hence, a series of standards containing L-tyrosine (Merck) in quantities of 0-300 ug per reaction volume (2.1 ml already containing 3.6 mg casein) was included in each assay. This was representative of digestion products as well as accounting for any spontaneous casein hydrolysis, and thus was used to quantitate proteolytic activity. One unit of proteolytic activity was defined as that amount of

active enzyme(s) extractable from one litre of cell suspension culture that will release, from casein, TCA-soluble material equivalent to 100 ug tyrosine under reaction conditions defined above.

Conditions influencing protease activity: The modified FCC protease detection method was evaluated for variation with reaction pH and temperature of incubation. One extract from a large cell suspension culture of fig was used for all test conditions, stored frozen 2 days between the pH series and the temperature series. The influence of pH was tested using buffers at pH 5.5, 6.0, 7.0 and 8.4. At pH 6.0, the following temperatures were tested: 27, 38, 47, 54 and 67 °C. Appropriate series of standard ficin and tyrosine were included with each set of conditions.

A number of reagents which could potentially activate the extracted enzyme(s) were tested under standard conditions, 40 °C and pH 6.0, with three controls. These controls were trypsin inhibitor (Sigma) at 0.4 mg/ml reaction mixture, sodium tetrathionate (ICN) at 6 mM final concentration, and no adjunct. The potential activators tested were as follows: L-cysteine-HCl (MCB) at 1.4 mM, dithiothreitol at 2.0 mM, EDTA at 2 mM, glutathione (MCB) at 6 mM, SDDC (sodium diethyldithiocarbamide, MCB) at 6 mM, calcium thiocyanate (Anachemia Chemicals, Ltd.) at 2.5 ul/ml reaction mixture, and thiourea (Mallinkrodt) at 6 mM final concentration. Both papain and ficin were tested in the presence of these reagents, and compared to the behaviour of the fig cell extract.

Milk clotting activity: Papaya and fig cell suspension cultures were harvested by filtration through Miracloth. Collected cells were lyophilized, weighed and extracted according to the methods described above. Extracts were kept on ice and used within 2 hr. The milk clotting assay of Balls and Hoover (1937) was used, with no results, so the following

method was devised. Skimmed milk was diluted to a solids content of 5% and the pH adjusted to 5.6 with 2 M H_3PO_4 then autoclaved in 25-ml Erlenmeyer flasks containing 3 ml each. Cell extract, 0.3 ml, was added to each of three flasks and these were incubated in a water bath shaker at 35°C up to 24 hr. Clotting times were compared to highly diluted standard papain, at a maximum concentration of 0.3 mg/ml.

Electrophoresis: Electrophoresis of ficin-like proteins in crude cell extracts in acrylamide gel was attempted. The method of Melachouris (1968), as modified by Mr. T. Kuwata (unpublished), was applied to a vertical slab gel apparatus. The separation gels tried were 9-10% acrylamide (Bio-Rad) polymerized with 0.24-0.28% bis-acrylamide; the concentration gels were 3-3.6% acrylamide; the electrophoretic buffer was Tris-TEMED-glycine at pH 8.9. After electrophoresis (4.5-5 hr 100 V), gels were fixed in TCA-isopropanol-water (15% TCA and 25% isopropanol in distilled water) for 40 min, stained with amido black 10B (0.025% w/v in 4:5:1 H_2O :methanol:acetic acid) 30-50 min, and destained 1.5-2 days in 3 changes of destaining solution (9.71:3.57:1 H_2O : methanol:acetic acid).

The disc gel method of Weber et al. (1972) was also employed, using 7.5-9.3% acrylamide. Electrophoresis was conducted in 0.1 M phosphate buffer (pH 7.2) with 0.1% SDS (sodium dodecylsulfate, Fisher). Gels were subjected to 2 mA per gel 20-30 min then 5 mA per gel until the bromophenol blue marker dye approached the distal end. Gels dislodged from glass tubes were stained up to 2 hr with Coomassie Brilliant Blue-G (0.25%, w/v, in 1:1:0.2 H_2O :methanol:acetic acid) and destained 1.5-2 days in 35:3:2 H_2O :acetic acid:methanol.

Samples were prepared for electrophoresis in a variety of ways in search of one that would effectively demonstrate proteins present in cell

extracts. For electrophoresis without SDS, sodium tetrathionate, SDDC and Triton X-100 were added, alone or in combination, to the extraction buffer in some trials. Many samples were concentrated in dialysis tubing (Fisher), packed in Carbowax 20M (Applied Science) 2-6 hr. For electrophoresis with SDS, samples were mixed with SDS, 8 M urea, and β -mercaptoethanol and boiled 5 min in stoppered tubes according to the method of Deutch (1976). Standards used were ficin and trypsin (Sigma). Samples and standards for all electrophoretic trials were mixed 4:1 with glycerol prior to application to gels.

4. Medium supplementation

Five groups of nutrient supplements were investigated with respect to enhancement of protease production of activity in cell extracts: inorganic nitrogen, amino acids, proteins, milk and its various components, and miscellaneous organic compounds, including two antibiotics, two thiol reagents and SDDC, an oxidase inhibitor.

Inorganic nitrogen and amino acids: Nitrogen is present in the standard B5 medium as KNO_3 (2.5 g/l, or 25 mM) and $(\text{NH}_4)_2\text{SO}_4$ (0.134 g/l, or 1 mM). Media were prepared omitting either of these, but substituting KCl or Na_2SO_4 as appropriate to maintain the non-nitrogenous ions (Behrend & Mateles, 1975). To each of these was added one of the following: alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine or proline (all L-isomers from Sigma) at 5 mM final concentration. For comparative purposes, a medium containing one-half the standard nitrate (12.5 mM) was prepared, and 3% (v/v) skimmed milk added to a portion of each nitrate-ammonium combination. Small flasks of these media were inoculated simultaneously from one suspension culture of Ficus carica grown in a

low-nitrogen medium, 20% of the normal B5 level. Following 9 days' incubation these conditioned cultures were used to inoculate another set of the same media for 14 days' incubation prior to assessment. Cells were harvested and both cells and media lyophilized and analyzed for protein content and protease activity, as described above.

Proteins and peptides: B5 medium was supplemented with a selection of proteins and related materials to investigate their possible stimulation of protease activity. These were as follows: casein powder (Fisher) at 0.3% (w/v), soy protein isolate ("Farefax", Nu-Life Nutrition) at 0.3% (w/v), wheat gluten ("WhetPro", Industrial Grain Products) at 0.3% (w/v), beef extract (Difco) at 0.3% (w/v), gelatin (MCB) at 0.3% (w/v), fresh egg albumen at 1% (v/v), enzymatic casein hydrolysate (ICN) at 0.3% (w/v), and skimmed milk at 3% (v/v). Two controls included were soluble starch (Difco) at 0.3% (w/v), and no supplements. All flasks were harvested after 18 days. Both media and cells were analyzed for protease activity and these values summed for total protease.

Milk and its components: Skimmed milk was used to supplement B5 medium for suspension-cultured cells of papaya and fig. This enriched medium was compared to standard B5 with respect to biomass, protein and protease activity over a growing period of 23 days under standard conditions of incubation. Assay procedures are described in preceding sections.

The effect of several components of skimmed milk on protease activity of fig cells was assessed by simple or complex supplementation of the medium. Single supplements, all reagent grade, were added at levels approximating that which 3% skimmed milk would provide for B5 medium: citric acid (49.8 mg/l), riboflavin (53 ug/l), niacin (24 ug/l) and calcium, as $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, (1.336 g/l in addition to that already present in B5).

Lactose replaced 20% of sucrose in one test medium, that is 4 g/l.

Commercial casein powder (Fisher) was rehydrated, precipitated and collected by centrifugation. Added at 0.3% (w/v), it was compared to fresh casein at the same level. These caseins were prepared by coagulation of agitated casein solution or fresh liquid skimmed milk with slow addition of phosphoric acid at room temperature until the pH reached 5.2. After 20 min continued stirring, the casein was collected by centrifugation at 10,000 xg for 15 min and the whey (supernatant) decanted off. After adjustment of the pH to 5.6 with NaOH, most of the whey was filtered through Whatman No. 5 paper and sintered glass (10-15 μ m), then subjected to ultrafiltration (Pellicon, Millipore) with a filter for 10,000 daltons. Both filtrate and retentate were used as B5 supplements, individually and in a 1:1 combination to a total of 2.7% (v/v). All media containing casein or whey prepared in this manner began with a basal medium containing 15% less initial phosphate, to accommodate the additional phosphate from the casein precipitation step. Flasks were inoculated from pre-conditioned cell suspensions grown 7 days in 20 ml of each respective test medium. After 14 days' incubation, cells were harvested and both cells and medium fractions analyzed for protein and protease activity.

Statistical analysis: Results of major experiments in medium supplementation were processed by analysis of variance using two programs prepared for the University of British Columbia computing centre, MFAV (Le, 1980) and Genlin (Grieg & Bjerring, 1980). To determine differences among treatments, Duncan's multiple range test was applied at probability levels of 1 and 5%. Data were grouped according to these ranges.

IV. RESULTS

1. Culture of plant tissues

Papaya tissues, C. papaya, were successfully cultured from excised segments of radicles and hypocotyls of axenically-germinated seeds. Early trials indicated that seeds germinated very poorly unless arils were first removed and seeds rinsed. The arils contain substances inhibitory to seed germination (Gherardi & Valio, 1976). Table IV includes survival levels of papaya cultures to both callus and cell suspension stages (Figure 4a & b). Calli were pale, soft and readily dispersable in liquid medium.

Fig tissues, F. carica, were successfully propagated from leaf segments to callus and suspension stages at the remarkably high survival level of 95% (Table IV). Fig suspension cultures were readily established and maintained, and were thus used for experimental studies in cell nutrition and enzyme production. The appearance of fig callus is illustrated in Figure 5 (a&b); cell suspension cultures were identical to those of papaya, consisting of a pale yellow slurry of cells.

Seeds of cardoon, C. cardunculus, showed a 14% germination rate, on average, and tissues were excised from hypocotyls and radicles for further propagation. Sterilization procedures used were not sufficiently effective for these hard seedcoats and a high rate of endogenous contamination resulted.

Galium, ladies' bedstraw, did not form callus from germinated seeds, but rather developed as a mass of root-like structures (Figure 6a). In attempts to bypass the stage of callus formation, germinated tissue was transferred to liquid medium. Under these conditions, the roots continued

Table IV Survival of Plant Tissue Cultures

plant source	explants type & number	% survival (2 mos.)	form in 6 mos.
<u>Ananas comosus</u>	fruit, leaf, stem 223	0	--
<u>Carica papaya</u>	100 seeds	22	callus & 11 suspensions
<u>Cirsium arvense</u>	leaf, petiole, stem 110	11	callus & 2 suspensions
<u>Cynara cardunculus</u>	leaf, petiole, stem 222	0	--
	72 seeds	22	callus
	40 seedlings	23	callus
<u>Dieffenbachia</u> <u>picta</u> & <u>amoena</u>	254 leaf & petiole	16 (all <u>D.amoena</u>)	little callus
<u>Ficus carica</u>	leaf, petiole, stem 133	95	callus & suspensions
	150 seeds	0	--
<u>Galium verum</u>	77 leaf & petiole	11	roots?
	81 stem	63	roots?
	19 seeds	0	--

a



b



Figure 4. Dark-grown papaya callus and cell suspension cultures.
(a) Papaya callus on B5 agar, 22 days after transfer.
(b) Papaya cell suspension culture, 18 days after transfer.

a



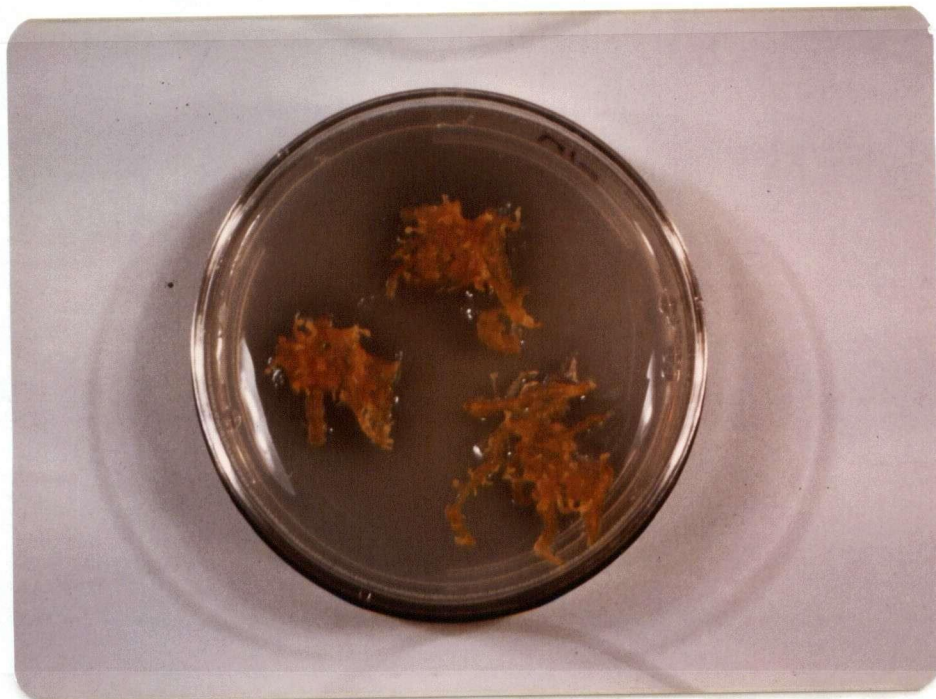
b



Figure 5. Fig callus development.

- (a) Fig callus formation on primary explants, 12 days after excision from leaf laminae and petioles.
- (b) Dark-grown fig callus after 4 generations (transfers).

a



b

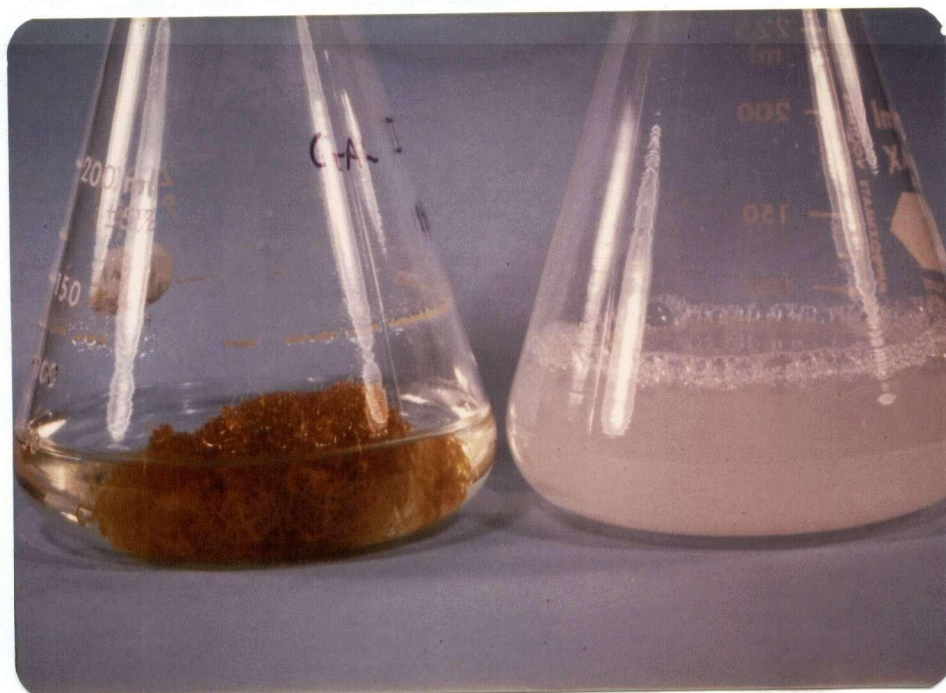


Figure 6. Dark-grown bedstraw tissue on agar and in liquid B5-M.

(a) Bedstraw growth on B5 agar medium, 35 days after transfer.

(b) Growth of non-callus bedstraw tissue in liquid B5 medium containing 3% skimmed milk. Flask on right is uninoculated B5-M.

to develop, forming large tangled masses as a result of rotary shaker action (Figure 6b). Milk-containing medium illustrated proteolytic activity. The medium was checked microscopically for sloughed cells which may have been used for single cell suspension, but these were very few in number. They did not proliferate when transferred to fresh medium, suggesting that they were actually dead cells, possibly sloughed from the rootcap area of developing roots. The survival rate of cultures is given in Table IV.

Tissues of dumbcane (Dieffenbachia), pineapple (Ananas) and thistle (Circium) all suffered high rates of microbial contamination in several trials (Table IV). Thistle explants formed some callus which was propagated for many months but grew slowly and would not grow as single cell suspensions. Dumbcane and pineapple explants callused very poorly and neither formed sufficient tissue to attempt cell suspension cultures.

Variability among cell suspension cultures: Fig and papaya cells grown in cell suspension exhibited differences not necessarily related to environmental conditions. It is likely that genetic factors influenced growth and production of protein and protease (Mandels, 1972; Skirvin, 1978). Figure 7 (a&b) shows yields of protein and protease in cell extracts from four papaya and fig batch cultures grown two weeks in B5-M medium prior to harvest. There was approximately a 3-fold difference in protein content and a 4-fold difference in protease activity among these four papaya cell cultures: the corresponding differences were 4-fold and 5-fold, respectively, in the four fig cell cultures. Although protease activity may have been correlated with protein production of papaya and fig cells, culture #1 of each, for example, this was not necessarily true (e.g., papaya culture #2). This variability existed despite similar propagation histories and use of the same medium and incubation conditions. Unfortunately, such

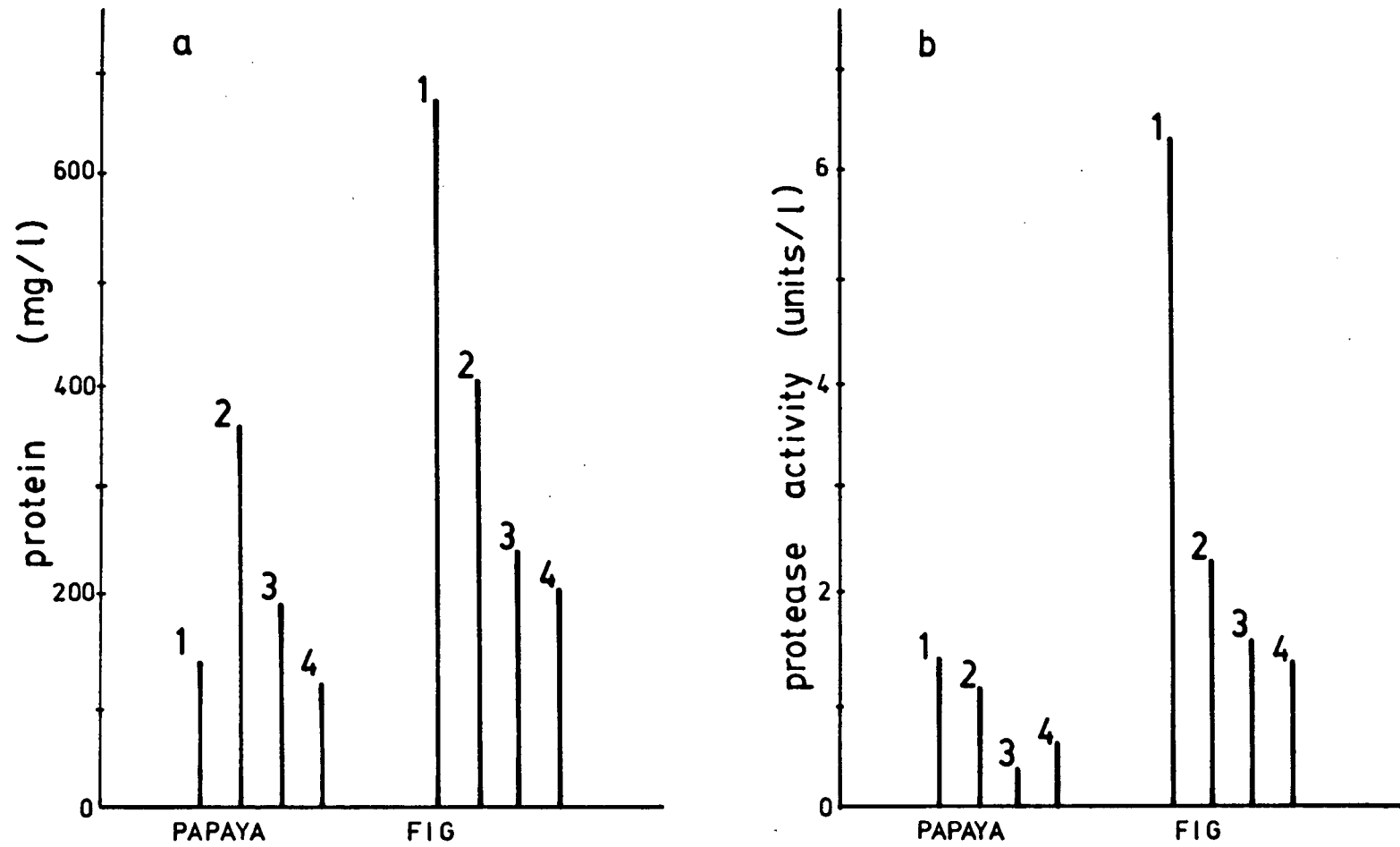


Figure 7. Protein and protease variability among cell extracts from four papaya and four fig cell suspension cultures. All samples were grown 13-17 days in B5 medium with 3% skimmed milk.

differences did not appear to be persistent. Figure 8 is a compilation of protein and enzyme activity data from some papaya and fig cell suspension cultures, but does not represent a specific experiment in itself. Protein and enzyme productivity did not appear to be correlated with number of transfers, since some consecutive cell suspensions showed an improvement while others declined. Again, protein synthesis and enzyme activity did not necessarily change together. With this degree of variability in culture productivity, it was essential to use one inoculum of a standard volume for each experimental set, as a control against changes with number of transfers and inoculum age. Judging from the variations apparent from one generation to the next, heredity alone was probably a minor factor. Other genetic factors such as nucleic acid synthesis, repair, regulation and foreign inclusions like plasmids or viruses were probably responsible for much of the variability noted. An investigation of genetic controls influencing cell growth and production of protein and protease in cell suspension was beyond the scope of the present work.

2. Assessment of assay methods

Cell growth and biomass production: Growth, as indicated by weight of harvested cells, is the simplest indicator of appropriate nutrition. Since there are several methods of assessing growth, some of these were compared. Table V gives harvest weight data from both papaya and fig suspensions as determined by freeze-drying, oven-drying and vacuum-oven drying. Results were reproducible for each method and indicated, further, that the two oven methods were the same (Students's t-test, $\alpha=0.05$), with lyophilized papaya samples weighing 10% more and lyophilized fig cells weighing 6% more (ie., containing that much more water after reaching a constant weight). These

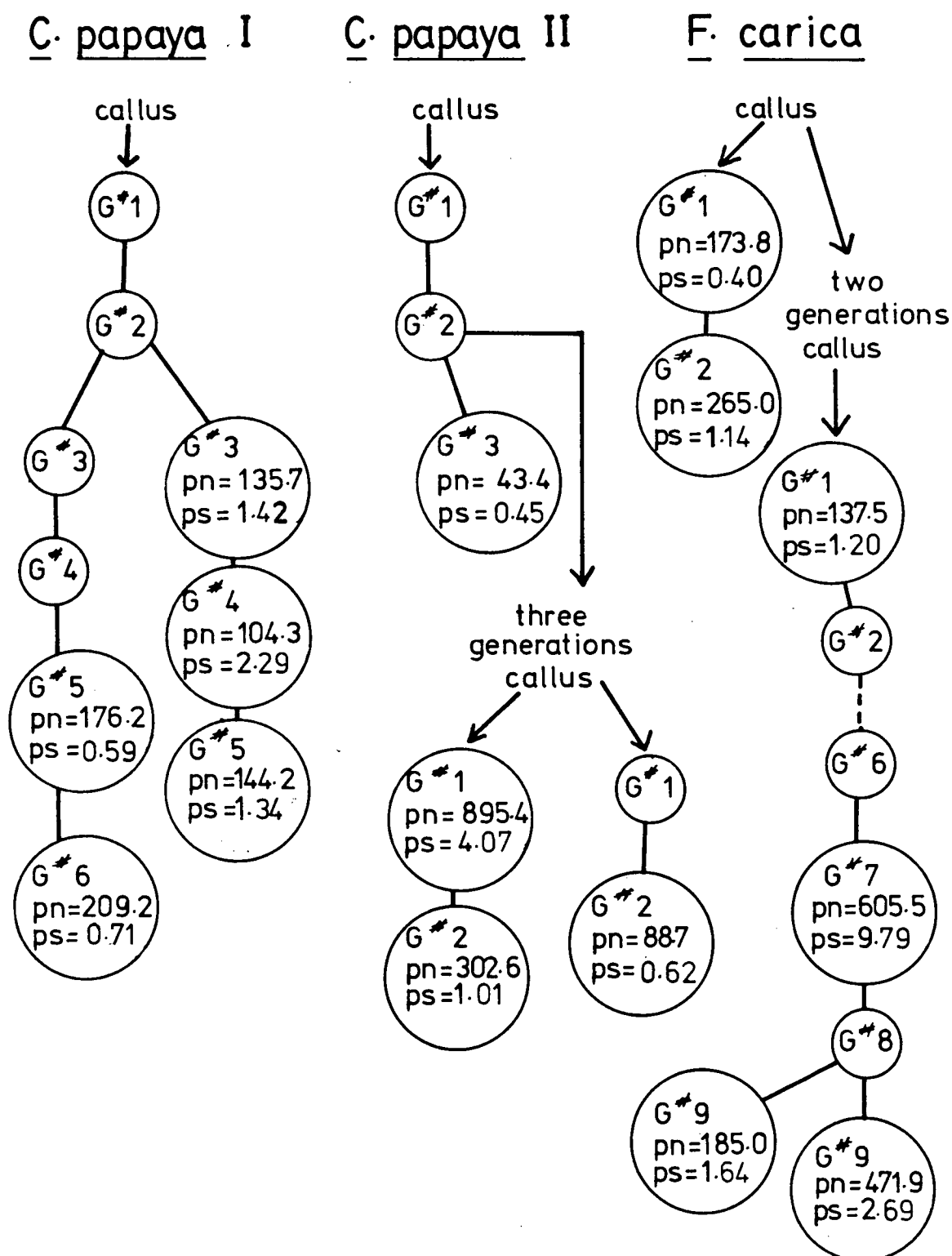


Figure 8. The role of heredity in variability of productive capacity: a compilation of data from one fig and two papaya cell cultures. G# = generation number in suspension culture; pn = protein content (cells + medium), (mg/l); ps = total protease (units/l).

Table V A comparison of drying methods for papaya and fig cells from suspension cultures. Each was initially 1.800 g blotted wet weight. n = number of samples; s = standard deviation of the mean.

drying method	papaya		fig	
	mean dry weight (mg)	s	mean dry weight (mg)	s
convection oven 60 °C (n=8)	17.7	0.46	14.9	0.58
vacuum oven 60 °C (n=8)	17.1	2.07	15.3	0.46
freeze dryer (n=6)	19.2	0.75	16.0	0.45

differences must be recalled in considering protein and enzyme data, as tissues were always lyophilized upon harvest to avoid problems of enzyme denaturation or hydrolysis of endogenous protein.

Settled cell volumes correlated well with cell dry weights up to about 30 ml cells, obtained from 50-ml cell suspension cultures (Figure 9). Above this, the curve reached a plateau indicating that large differences in settled cell volumes were not necessarily weight-related. This may have been due to difficulty of cell packing in the restrictive vessels used, 50-ml centrifuge tubes.

Typical growth curves, on the basis of lyophilized weights, for papaya and fig cell cultures grown in B5 medium are presented in Figure 10. The logarithmic growth phase began within four days, with biomass reaching a maximum in 17-21 days. There was an initial drop in medium pH, which then began to climb after the fourth day, reaching values more basic than the initial medium. With fig and papaya cultures, approximately equivalent biomass in B5 medium, about 3 g/l, were produced.

Cell extraction: Table VI gives protein yields and enzyme activity of extracts made using a Polytron homogenizer, a Braun sonicator or a mortar and pestle. No distinct differences were evident in protein yields or enzyme activity of homogenized or mortar-ground samples. Sonication did not appear to be as effective in protein extraction, and thus also showed lower protease activity. Only a single sample was prepared by each method, though assayed in duplicate, since a large volume of cells was required in order to do the comparison on a single culture. It is therefore possible only to speculate on the differences seen: 150 sec homogenizing resulted in lower enzyme activity than the shorter time, sonication showed lower protein extraction and protease activities than other methods. Where cell yields,

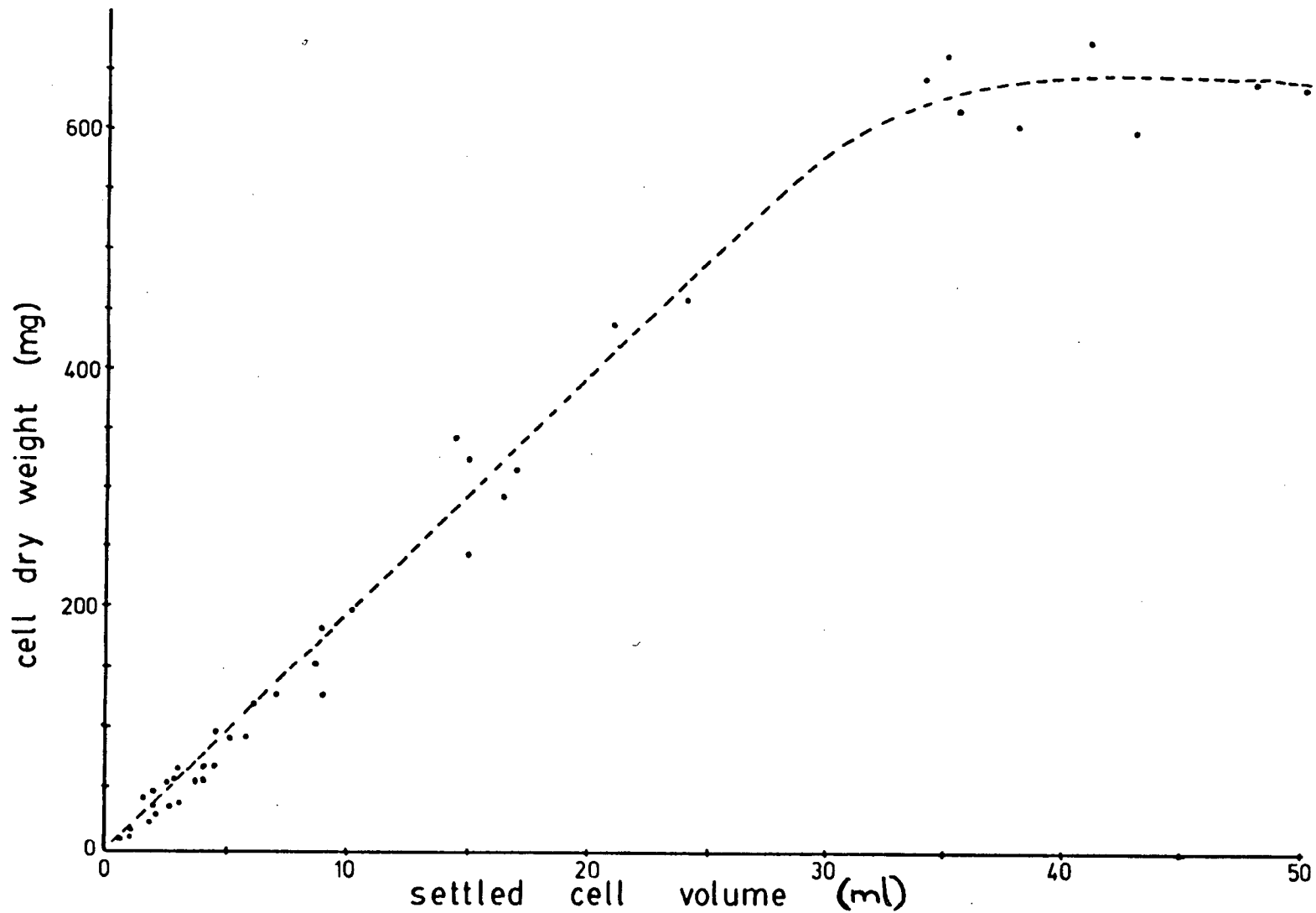


Figure 9. Settled cell volume as an indicator of harvest weight. Harvested fig cells were used to determine the relationship of settled cell volume to cell dry weight (lyophilized).

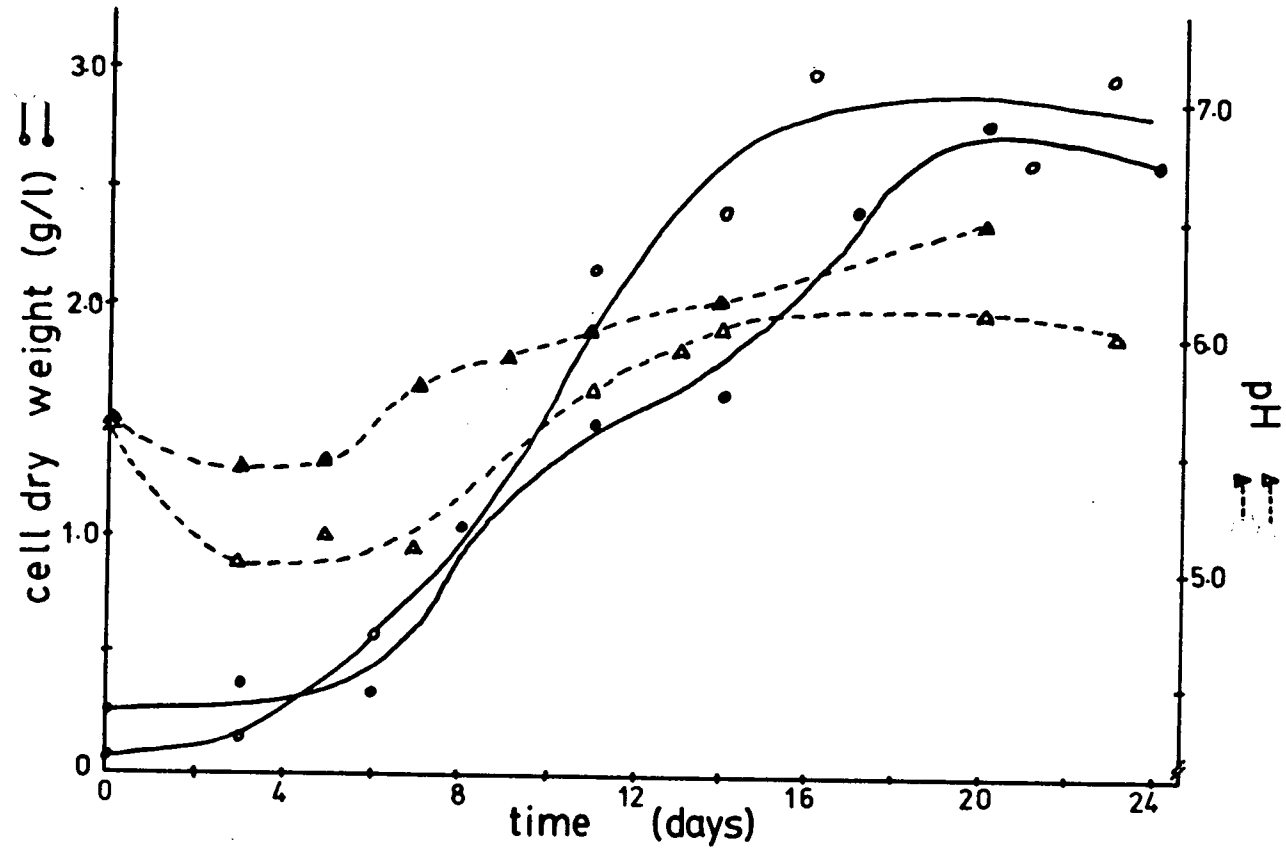


Figure 10. Growth of papaya and fig cell suspension cultures in B5 medium: biomass and pH changes. open symbols, fig; closed symbols, papaya; dry weight from lyophilized cells.

Table VI Liberation of protein and active protease from fig cells by different extraction methods. Each sample consisted of 0.545 g cells (dry weight), and all were ground with 0.1 M phosphate buffer, pH 6.0.

extraction method	extraction time (sec)	protein ¹ (mg/g dry cells)	protease activity ² (mg/g dry cells)(mg/g protein)	
mortar & pestle	90	15.5	6.3	0.50
mortar & pestle with sand	45	11.4	7.1	0.62
	90	14.2	6.7	0.47
Polytron homogenizer	120	10.4	7.1	0.68
	240	10.7	5.7	0.54
Braun sonicator	30	9.5	4.8	0.51
	120	11.1	4.7	0.42

1 means of duplicate determinations using Bradford's protein assay.

2 means of duplicate determinations using the modified FCC method for protease. TCA-soluble digestion products were equated with quantities of L-tyrosine prepared in the same manner.

and thus extraction volumes, were small, the Polytron homogenizer and Braun sonicator both result in rapid heating of the solution, even with the use of an ice bath. This could activate enzymes earlier than desired, or cause enzyme denaturation. Because it is easier to grind cells while maintaining a low temperature using a mortar, this was the preferred method. All further cell extractions were conducted with ignited sand in a mortar kept on ice, using chilled buffer (4-8 °C).

Protein quantitation: Bradford's method of protein quantitation is rapid and simple. Figure 11 shows a standard curve of absorbance at 595 nm plotted against protein content, using bovine serum albumen (BSA). A standard curve was produced for each protein assay and results determined by linear regression. This precaution circumvented possible variability introduced by any change in the reagent or experimental conditions. Fresh reagent was prepared fortnightly as longer storage resulted in a loss of sensitivity. The protein content of cell extracts was found to be in the range of 2.5 to 7% of dry weight.

Other methods of protein assay were less effective and/or more cumbersome. The Lowry procedure required higher concentrations of standard protein (BSA) and thus, was not sensitive enough for detection of protein in extracts from small cell harvests. Because of turbidity and the spectrum of compounds present in crude cell extracts, simple absorbance readings with 280 nm light cannot be assumed to reflect protein content alone, so this method was rejected.

Nitrogen content has often been used as a measure of protein. Kjeldahl digestion gave results ranging from 27.4 to 46.5 mg nitrogen per g weight of dried fig cells, averaging 3.58% for cells grown in B5 medium. This would indicate an average total protein content of 22.4% (n=20, s=2.98)

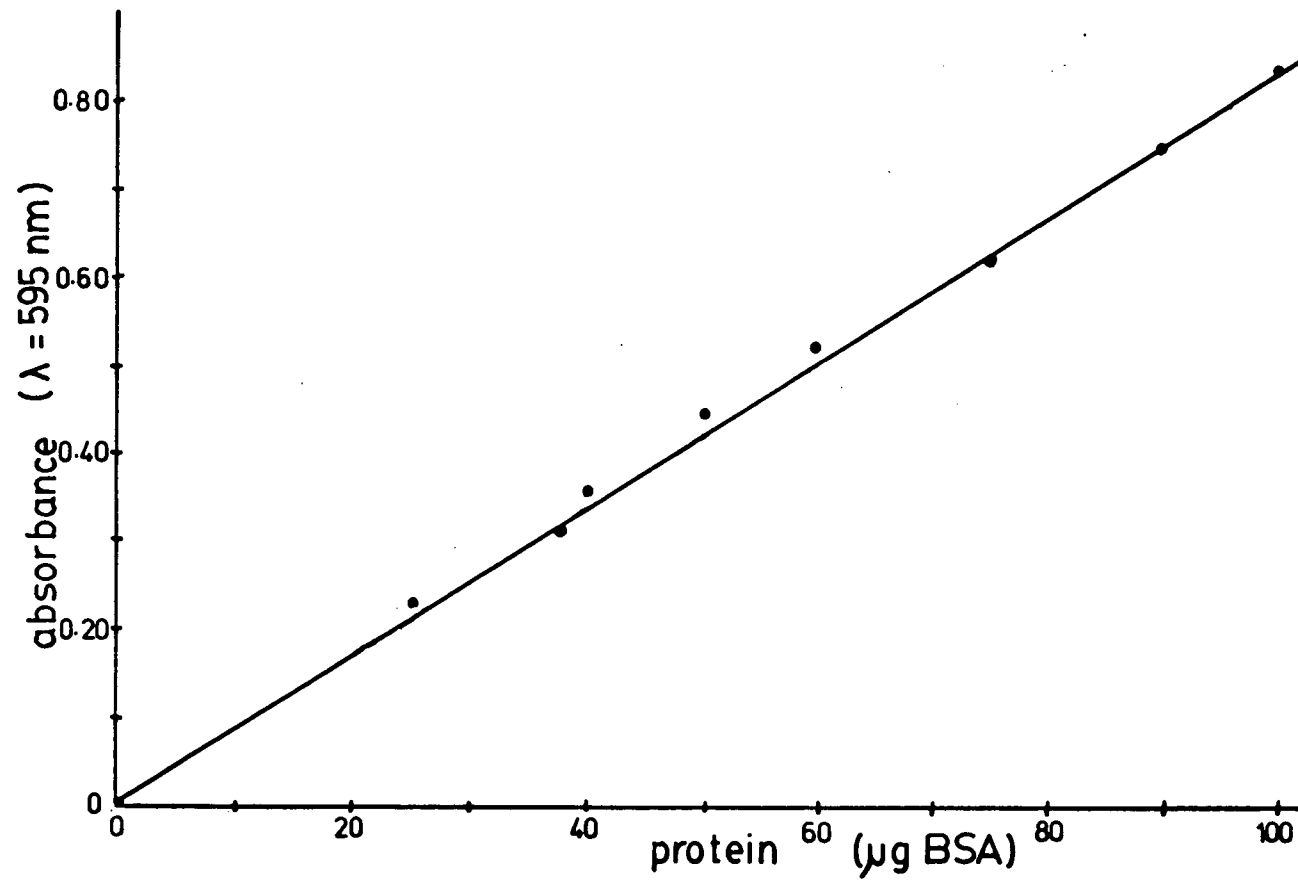


Figure 11: Bradford's protein assay: typical standard curve using bovine serum albumin (BSA). This plot fits the regression equation $y = 0.01x + 0.02$ ($r^2 = 0.99$).

if the average protein nitrogen factor of 6.25 was applied. However, these nitrogen determinations were based on digestion of entire cells, whereas protease assays were conducted on cell extracts. This method was deemed inappropriate for routine use in extract digestion because of the time involved and the possibility of variation in the ratio of nucleic acids to intracellular proteins or amino acid pools during development of individual cells.

As determined by Bradford's method, extracts of fig cells contained an average of 5.56% protein ($n=28$, $s=1.65$) in rapidly-growing cell suspension cultures two weeks after transfer. At the same time, in B5 medium without milk, the average protein content of fig cell extracts was 3.53% ($n=14$, $s=1.33$). These two figures are much lower than that determined by Kjeldahl digestions since this method omits insoluble proteins in disrupted cells, and does not encompass nucleic acids and other non-protein nitrogen.

Protease quantitation: The clearing of milk-containing agar was the earliest indication of proteolytic activity in plant tissue cultures. This was evident in explants and callus tissues of papaya, fig, cardoon, thistle and bedstraw. Cell suspension cultures of papaya and fig, and liquid-grown bedstraw also demonstrated clearing of milk-containing medium. Casein was evidently a suitable substrate, so this phenomenon was used as an indicator in the selection of assay methods. This decision tended to lead into potential dairy applications, whereas a meat-digestion assay method such as that devised by Orsi and Major (1973) would have led to an investigation of meat-industry applications.

Gel diffusion methods of enzyme detection proved ineffective for cell extracts. Araki and Abe's method (1980) relies on a localized zone of

colour change in the agar medium containing BAEE substrate. No zones indicating esterolytic activity could be detected surrounding wells containing cell extract, though papain was detectable down to 0.1 mg/ml. The BioRad gel diffusion test is also performed in agar, but contains casein as the substrate. The diffusion zone is seen as a clear area surrounding protease-containing wells. Again, no zones around cell extracts were evident under the specified assay conditions, although standard ficin was detected as low as 10 ug/ml in the presence of dithiothreitol. The agar diffusion method of Holmes and Ernstrom (1973) is also based on casein, but is performed in tubes (vertical diffusion), and results were similar to the BioRad method. It would appear that, if protease was present, conditions of these tests were not optimal for their diffusion through or activity in an agar environment.

Another protein substrate, collagen, was investigated by two methods. The azure-bound hide powder (Savage & Thompson, 1970) was slightly digested by an extract of fig cells, equivalent to 0.03-0.04 ug ficin per mg cell dry weight extracted. Film gelatin (Glenister & Becker, 1961) was also digested by enzymes in the crude fig cell extract, equivalent to 0.059 ug ficin per mg extracted cells. These are both at the low end of the range of ficin concentrations with detectable activity.

None of the above methods permits comparison with other proteases without preparation of a standard curve for each enzyme of interest. That is, each assay of papaya cells would require a dilution series of standard papain, and fig cells would require a similar series of the ficin standards.

Fluorometric methods of protease detection tested gave unsatisfactory results. The method of Spencer and Spencer (1974) appeared to work well with chymotrypsin, but was subject to complications when used

with sulfhydryl proteases. ANS fluorescence increased sharply upon addition of the cell extracts, possibly due to interaction of -SH reactive groups with the casein substrate. Fluorescence did not subsequently decrease. This complication, and the lack of any evidence indicating proteolytic activity in crude cell extracts, discouraged further use of direct fluorimetric methods. The procedure given by Chism et al. (1979) calls for pre-incubation of the sample with the casein substrate, prior to TCA precipitation and fluorescamine addition. There was a great deal of variability among samples containing less than 1.0 unit/ml of papain.

The FCC method for papain was adopted for routine quantitation of protease activity, modified as described in section III-3. This method proved to be the most reliable and sensitive one that could conveniently be applied on a routine basis. One advantage of the FCC method is that final data are derived from absorbance readings at 280 nm and can readily be related to quantities of a single aromatic amino acid. Tyrosine is most commonly reported in the literature, so it was used to prepare a standard curve for each assay, as illustrated in Figure 12. Cell extracts often contained NPN soluble in 10% TCA which produced absorbance readings equivalent to as much tyrosine as 1 mg/ml. Thus assessment of true proteolytic action on the casein substrate was based on subtraction of absorbances of controls from absorbances of incubated samples. By linear regression these differences were related to the standards to give equivalent amounts of tyrosine which would have been released from casein by proteolytic activity. If aromatic amino acids, tryptophan, tyrosine and phenylalanine comprise approximately 15% of casein, then results of 80 ug of tyrosine released would indicate 1.2 mg of amino acids released from casein, assuming all amino acids are released equally.

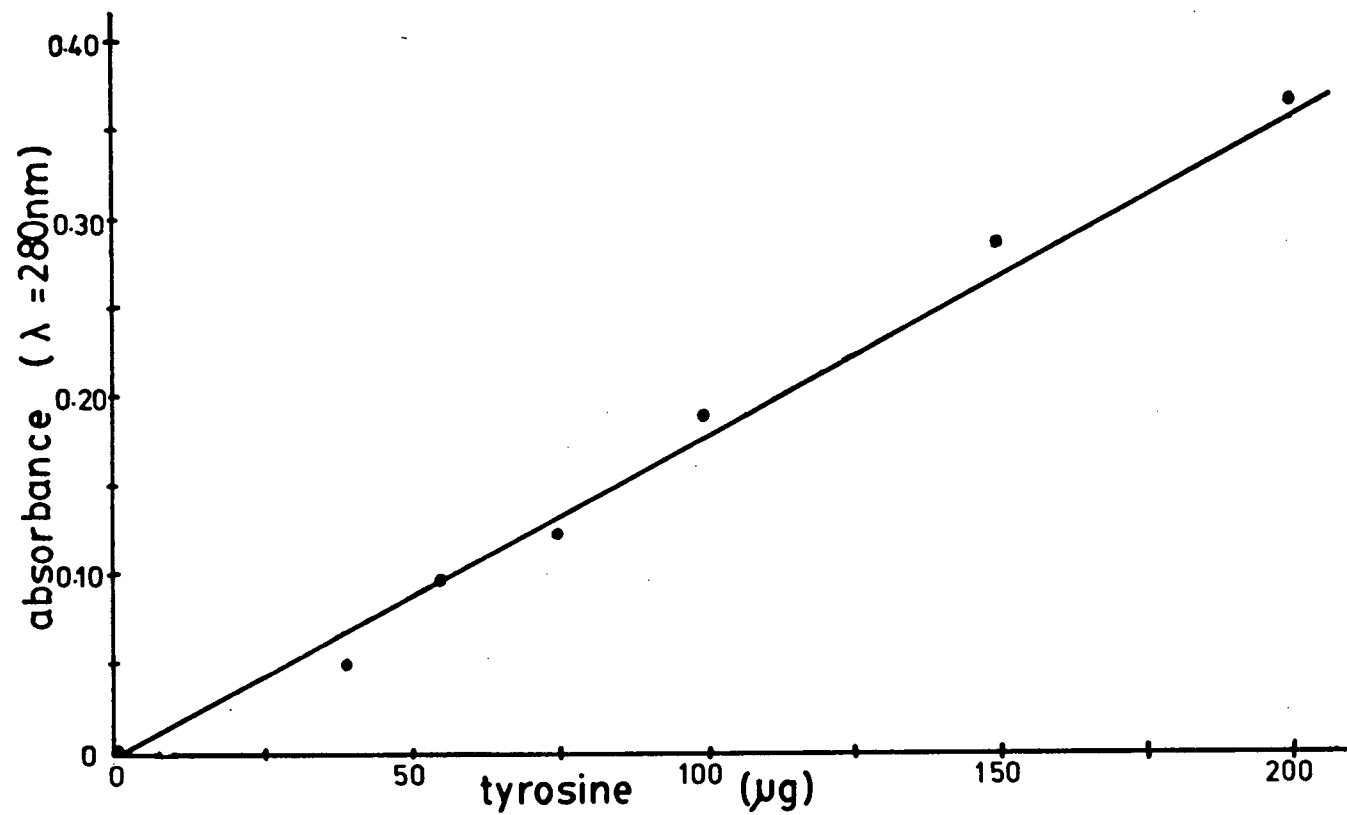


Figure 12. Typical protease assay standard curve using L-tyrosine. This plot fits the regression equation $y = 0.0018x - 0.01$ ($r^2 = 0.99$).

Factors influencing protease activity: The effect of pH on protease activity at 38 °C of ficin and fig cell extract is illustrated in Figure 13a. Although ficin activity at this temperature remained relatively constant over the pH range 5.5 to 8.4, the fig cell extract demonstrated a clear pH optimum about 6.0. Temperature also had little effect on the standard ficin preparation over the range of 28-67 °C, whereas the fig cell extract showed a temperature optimum around 47 °C (Fig. 13b).

The influence of several compounds on ficin activity at 38 °C is shown in Figure 14. Two concentrations of ficin were tested with each compound. Only cysteine and dithiothreitol (with or without EDTA) had pronounced stimulatory effects, while thiocyanate, sodium tetrathionate and trypsin inhibitor were inhibitory. Glutathione, EDTA alone and SDDC resulted in a slight increase in absorbance over the base levels, which contained no ficin. Protease activity of the fig cell extract was not affected greatly by any of the test compounds, but was stimulated slightly by Dtt, with or without EDTA. SDDC also appeared to be stimulatory, but there was interference by colour development in those reaction tubes. Thiocyanate, sodium tetrathionate and trypsin inhibitor had essentially no effect on assay results.

3. Milk clotting activity

Clotting times of cell extracts were determined, according to the procedure in section III-3, for cell extracts from five cultures of papaya and one of fig. Results, given in Table VII, show the fastest clotting time was 5.5 hr (papaya #4). The only fig cell extract tested required 12 hr to clot milk casein. Also included in Table VII are clotting times required

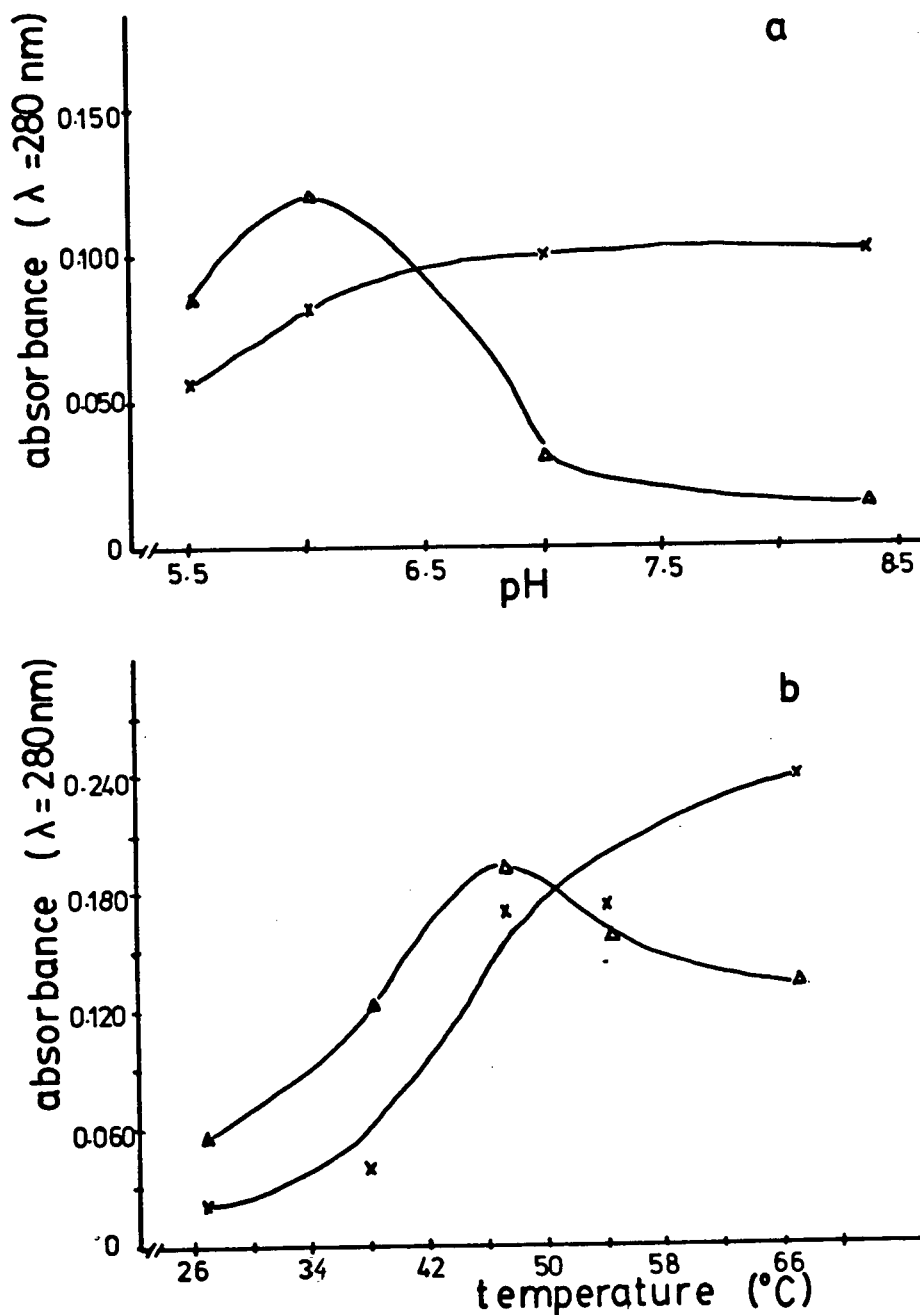


Figure 13. Effect of pH and temperature on activity of ficin and fig cell extract. Protease activity was determined by the modified FCC method (see text). Ficin was used at 0.42 mg/ml (x). Fig cells were extracted by grinding with 0.1 M phosphate buffer, (Δ).

- (a) Casein substrate prepared in buffers of different pH, all samples incubated at 40 $^{\circ}\text{C}$.
- (b) Casein substrate, pH 6.0, samples incubated at 27–57 $^{\circ}\text{C}$.

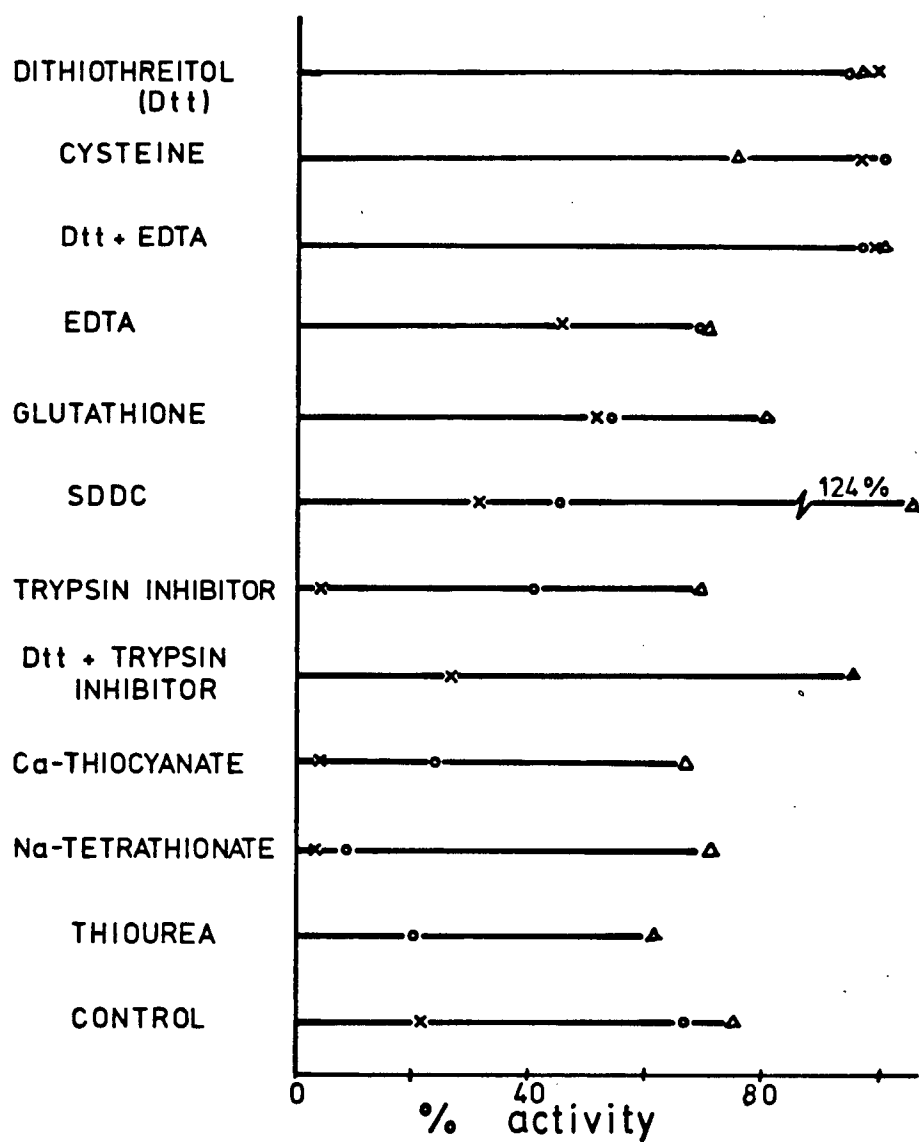


Figure 14. Influence of some activators and inhibitors on proteolytic activity. Papain (100 ug), cysteine designated as 100% of activity (○); ficin (21 ug), Dtt designated as 100% of activity (x); fig cell extract, Dtt + EDTA set as 100% of activity (Δ). SDDC is sodium diethyldithiocarbamate.

Table VII Milk clotting activity of plant cell extracts in comparison to standard papain. Five different papaya cell cultures and one fig cell culture (numbers corresponding to data in Figure 7) were extracted and tested for clotting activity as described in the text.

test material	clotting time ¹ (hr)
(a) cell extracts	
papaya 1	13.0
papaya 2	12.5
papaya 3	15.0
papaya 4	5.5
papaya 5	18.0
fig 4	12.0
(b) papain standards (ug/ml)	
16.3	3.5
8.2	5.0
5.4	10.8
4.1	14.0
0	> 24

¹ means of duplicate determinations

for a dilution series of crude papain. These standards indicate that the extract with the shortest clotting time possesses clotting activity equivalent to about 8 ug/ml papain, by linear interpolation. Others were even less active. Milk clotting activity was not assessed routinely so no data are available regarding other cell cultures such as later generations.

4. Electrophoresis

The greatest difficulty encountered in attempts at separation of proteins in cell extracts was concentration. Extracts normally used for protein and protease determinations were obtained by grinding lyophilized cells (in the form of a spongy mat) in a mortar with sufficient phosphate buffer to wet the tissue, and the thick slurries were then filtered. Extracts were generally found to contain 0.2-1.2 mg protein/ml. No protein bands were detectable by electrophoresis of these cell extracts. Passage of one sample through a small Sephadex G-25 column, equilibrated with extraction buffer, to remove compounds possibly interfering with protein mobility did not appear to help. SDS-electrophoresis of samples prepared by boiling with urea and β -mercaptoethanol was no more successful. The standard sample was a Sigma ficin preparation, twice recrystallized, containing 0.5 mg protein/ml. Over a period of 4-5 hr electrophoresis, there was sufficient movement to indicate three, possibly four, poorly-separated protein bands in the purified ficin. Trypsin was applied to the gel as a standard and was found to migrate more readily though it, too, showed three bands.

Only one attempt was made to locate proteolytic bands by contact of the finished, unfixed gel with Bio-Rad casein protease detection agar. No clearing was visible in 8 hr at 28 °C. Concentration of samples by

long-term contact with Carbowax 20 M (Applied Science, Pa.), 5-8 hr at 6 °C, samples being contained in dialysis tubing, was also inadequate. In many cases, flocculation of the concentrate occurred, and none showed improved mobility. It is postulated that proteins in cell extracts were physically bound to other extract components, or to the polyacrylamide gel itself, thus preventing their mobility in a gel environment while causing no interference with protein or protease assays, conducted in fluid environments. This suggestion would be supported by the negative results obtained with agar-based protease assays.

5. Medium supplementation

Four groups of nutrient supplements were investigated with respect to enhancement of protease production and activity in cell extracts: low molecular weight nitrogen sources, proteins, skimmed milk and its components, and miscellaneous organic compounds including two antibiotics. Each group of materials comprised a separate series of cell suspension cultures handled uniformly and each inoculated from a single cell suspension. Only fig cell cultures were used for these studies.

Nitrogen nutrition in fig cell cultures: The relative importance of the two nitrogen sources in B5 medium was determined using complete B5, B5 with half the normal nitrate concentration, B5 without nitrate (ammonium only) and B5 without ammonium (nitrate only). Each of these was also prepared with skimmed milk (3%, v/v). The results, presented in Figure 15, indicate that nitrate was the most important of the nitrogen sources. Biomass, total protein and total protease activity were all lowest in the absence of nitrate. Although milk went a long way toward correcting this deficiency in terms of biomass, it did not provide a significant improvement in protein or protease yield over the non-milk media where nitrate was absent.

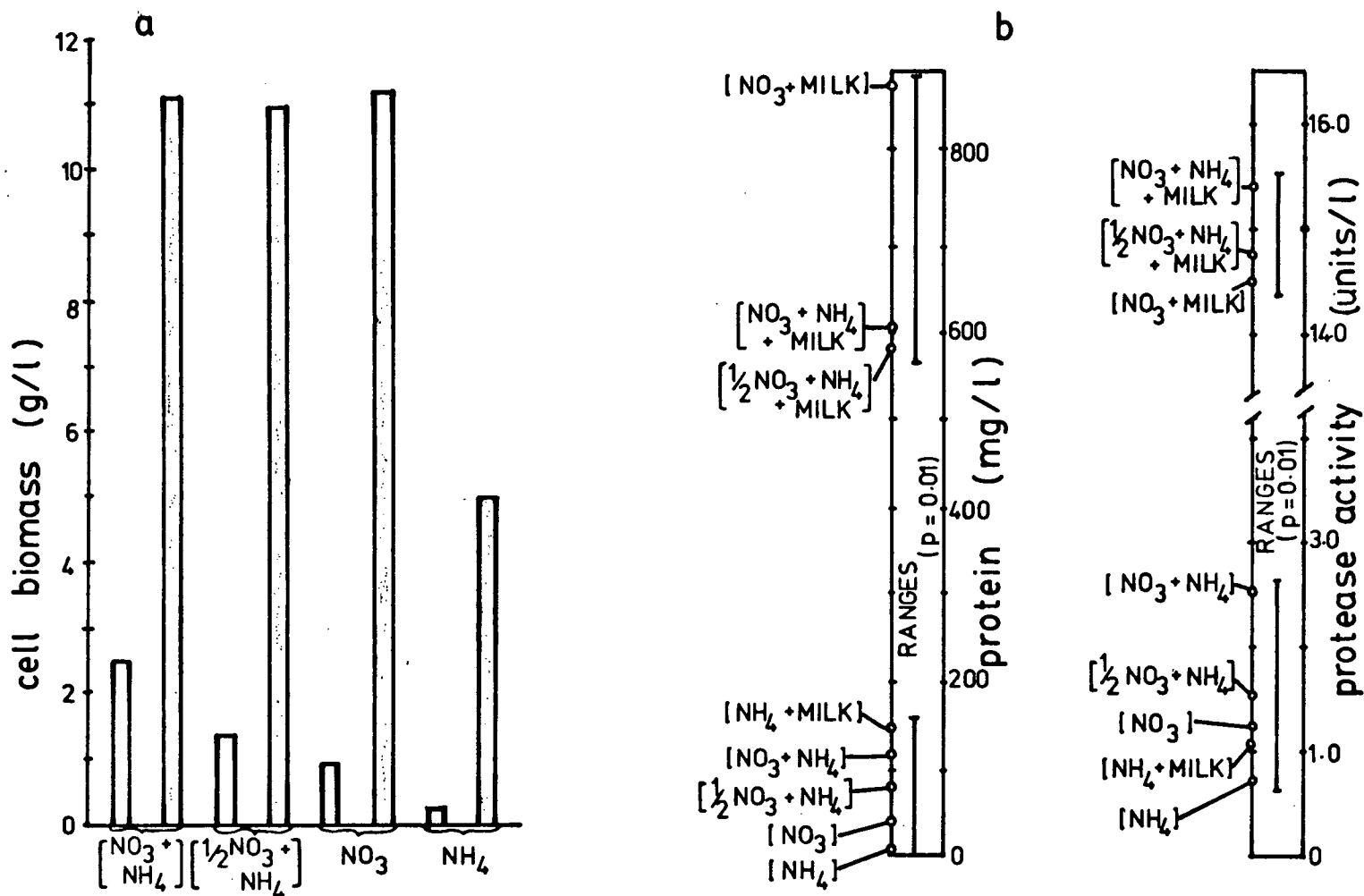


Figure 15. Inorganic nitrogen supply in fig cell cultures, and interaction with milk.

- (a) Effect on biomass. Shaded bars give cell weights from the same medium with the addition of 3% skimmed milk.
- (b) Effect on protein and protease activity. Range spans encompass groups of significantly different media, at the 1% level, using Duncan's multiple range test.

Several amino acids were added to incomplete B5 medium (only nitrate or ammonium) in search of keys to nitrogen metabolism that may be utilized to stimulate production of protein and active protease. Results of cell harvests (Figure 16) underlined the importance of nitrate as the base of nitrogen metabolism. In its presence, several amino acids stimulated growth while its absence produced generally poor growth irrespective of other supplements with the sole exception of milk.

Glutamate actually stimulated protein synthesis in non-nitrate medium to a level equivalent to unsupplemented nitrate medium, an adequate replacement for nitrate (Figure 17a). Stimulation of protease activity was most obvious with the addition of aspartate to nitrate-containing medium, with glutamate, cysteine and possibly arginine also causing a significant improvement in protease activity, relative to complete B5 (Figure 17b). With the ammonium-based medium, there were no outstanding significant differences among supplements with respect to protease activity. Duncan's multiple range test at the 5% probability level gave overlapping ranges over the entire data base. Only milk and cysteine were significantly better than arginine and alanine, all others falling between and overlapping these extremes.

These data indicate that the amino acids capable of stimulating protein synthesis were also apt to increase protease activity. With the additional case of cysteine, the inverse was also true, high protease levels indicating high protein levels. Cysteine at 5mM may have caused activation of enzymes normally produced by the cells, or actually stimulated enzyme synthesis. It is doubtful that glutamate and aspartate serve only enzyme-related functions, since they caused stimulation of total protein synthesis as well as an improvement in total proteolytic activity of

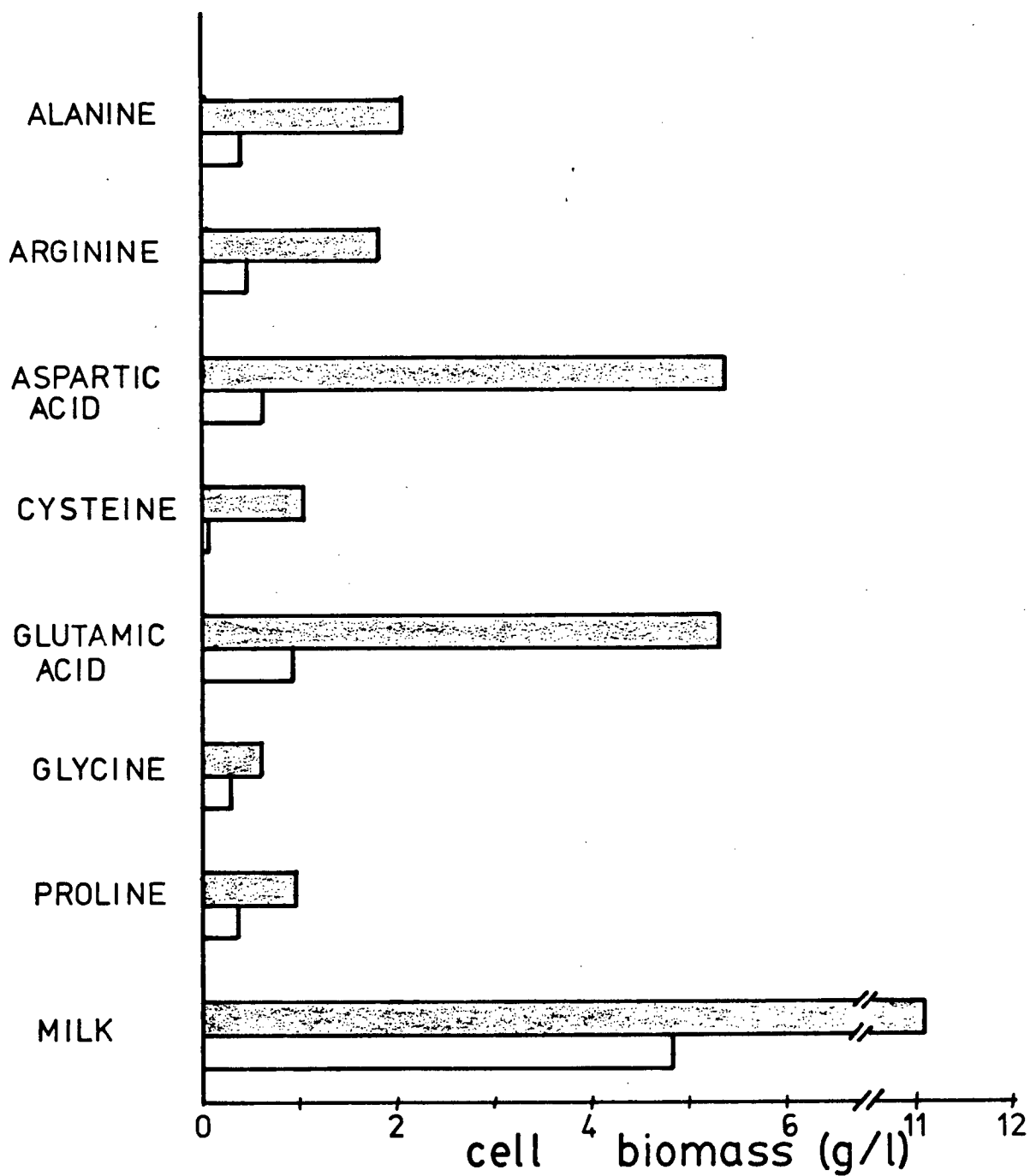


Figure 16. Amino acid supplements and skimmed milk: effect on biomass in fig cell suspension cultures. Shaded bars give cell weights from NO₃-based media, open bars from NH₄ media.

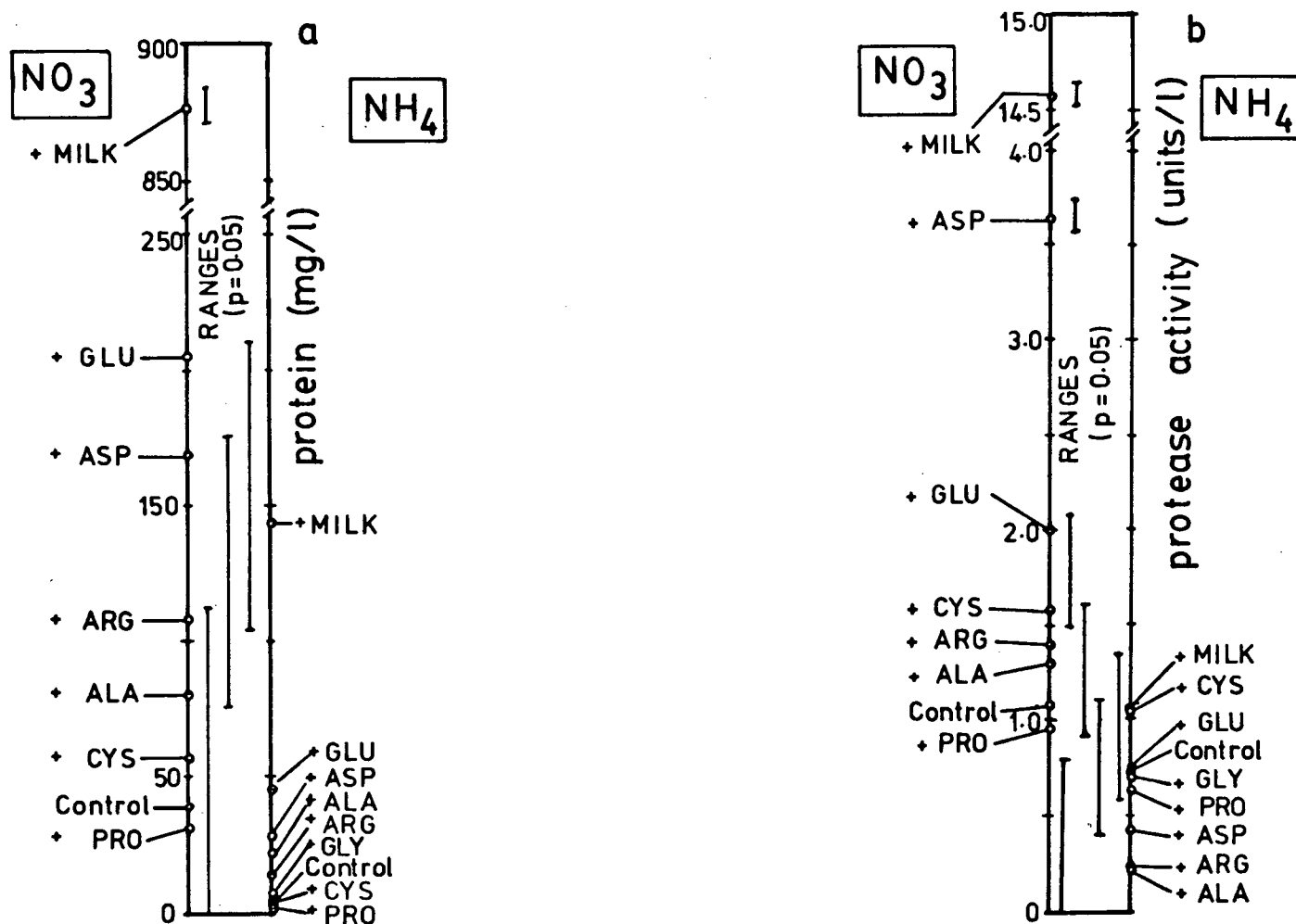


Figure 17. Amino acid supplements and milk: effect on protein and protease activity in fig cell cultures deprived of either nitrate or ammonia. Nitrate-based media are on the left and ammonia-based media on the right side of both (a) protein, and (b) protease. The control contained only NO₃ or NH₄ but no amino acids. Range spans encompass groups of all media differing from each other at the 5% level, using Duncan's multiple range test.

suspension-cultured fig cells.

Effect of proteins and peptides on fig cells: Protease activity of fig cell cultures was not stimulated by most of the proteinaceous materials tested. Results are given in Figure 18, including two control media, one with no supplementation and one containing soluble starch, a polymer of very different chemistry. Only milk and egg albumen resulted in protease activity levels significantly higher than B5 medium alone. On the contrary, beef extract, casein, wheat gluten, gelatin and casein hydrolysate produced significantly lower protease activity in fig cell cultures ($p=0.01$). Starch was also inhibitory. Causes of enzyme inhibition were not examined, though it is speculated that adsorption of proteolytic enzymes or associated ions by polymers could lead to interference with activity. Skimmed milk, being easier to work with in media than egg albumen, was selected for further study with respect to identification of stimulatory factors.

Effect of skimmed milk and its components on fig cells: The addition of 3% skimmed milk to B5 medium had a profound stimulatory effect on growth of papaya and fig cells. Typical protein and protease productivities are shown in Figure 19. Some of the supplements added to B5 medium in this study were derived directly from skimmed milk as described in section III-4. Others were pure compounds added at levels approximately equivalent to quantities found in milk. The results, Figure 20, showed a 10-fold spread in biomass yields from this range of supplements. Whey and the retained portion of ultrafiltered whey both produced growth which was at least equal to that obtained with milk. Casein and especially ultrafiltered whey were ineffective growth promoters. Of the simple supplements, only citric acid produced a good growth response, approaching that of milk itself.

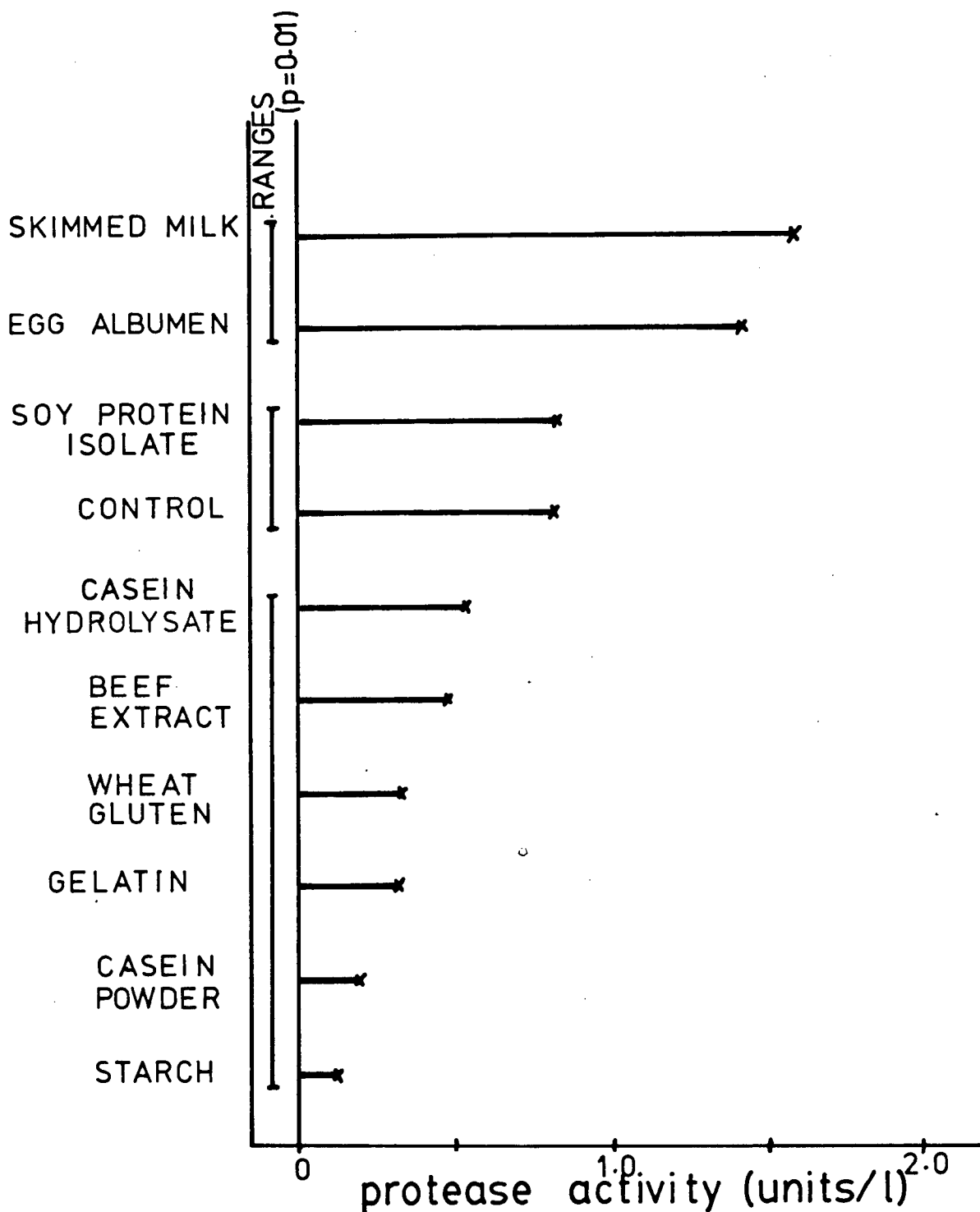


Figure 18. Proteins and peptides: effect on protease activity in fig cell cultures. Two controls were included, starch and unsupplemented B5 medium. Range spans encompass groups of differing at the 1% probability level (Duncan's multiple range test).

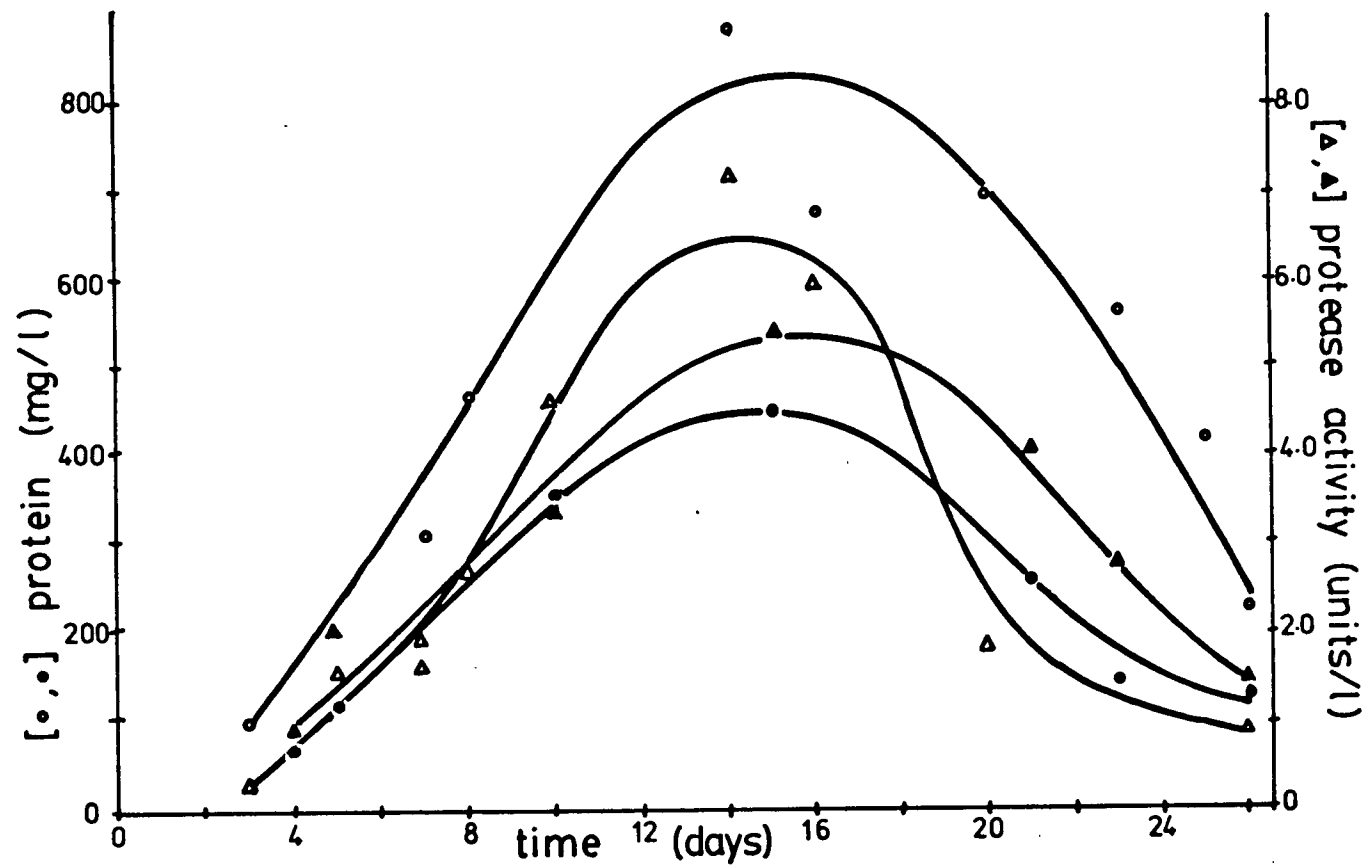


Figure 19. Protein and protease activity in fig and papaya cell suspension cultures over 3 weeks.
open symbols, fig; closed symbols, papaya

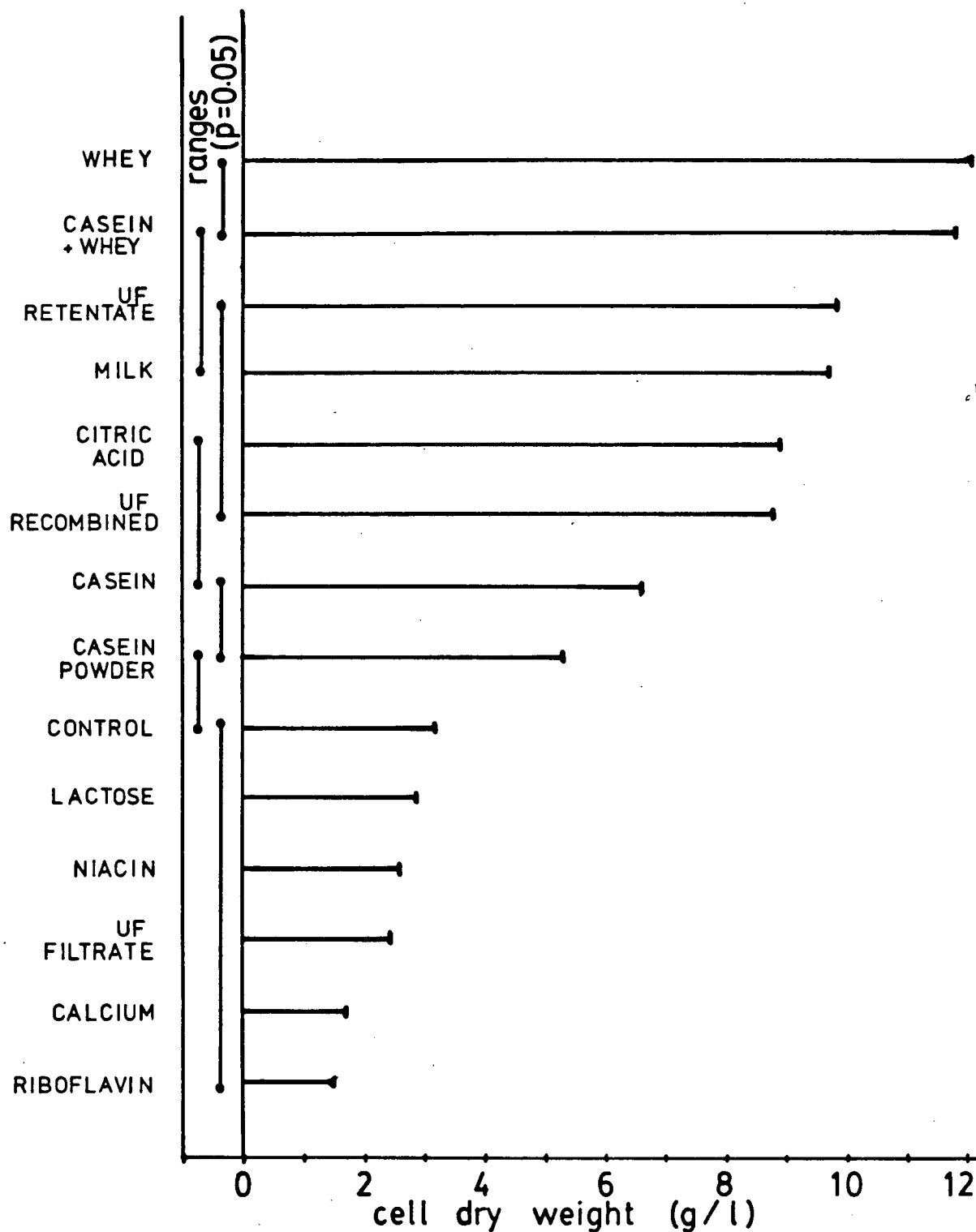


Figure 20. Skimmed milk and its components as supplements: effect on biomass. Fig cell cultures were grown in B5 containing the above supplements. UF refers to ultrafiltration products, retentate, filtrate or these recombined (1:1). Range spans encompass groups differing from each other, using Duncan's multiple range test at the 5% level.

Protein yield was highest with skimmed milk, whey and casein+whey (recombined), followed by ultrafiltration retentate, casein (fresh and powdered) and citric acid (Fig. 21a). Citric acid led to only 57% as much protein synthesis as whole milk, and the other supplements fell far short of this. Proteolytic activity, as shown in Figure 21b, was greatest when the milk ultrafiltration retentate was added to the medium. Milk, whey-containing media, fresh casein and citrate all produced significantly greater enzyme activity than other supplements. The unusually high protease activity achieved using ultrafiltration retentate was due to its addition at the same level as whole whey, although it actually comprised about two-thirds of the total whey volume. In effect, this resulted in the addition of 50% more of these components than are normally added in the skimmed milk supplement. The active component of whey was retained by the 10,000-dalton filter, so the ultrafiltrate had no stimulatory effect on yields of biomass, protein or protease. Recombined with retentate, intermediate results were obtained. The effectiveness of the retained fraction of whey was most notable in total protease activity, where it yielded 38% higher activity than skimmed milk. Although fresh casein and reconstituted casein powder produced similar cell harvests, 6.57 and 5.26 g/l respectively, fig cells grown with the latter demonstrated only about half the protease activity of those grown with fresh casein. Citric acid produced a significant improvement in protease activity relative to unsupplemented B5 medium ($p=0.05$). Enzyme activity in the citrate-supplemented fig cell culture released the equivalent of approximately 400 mg of tyrosine per litre of 14-day cells, a level not significantly different from that obtained with skimmed milk, casein or whey.

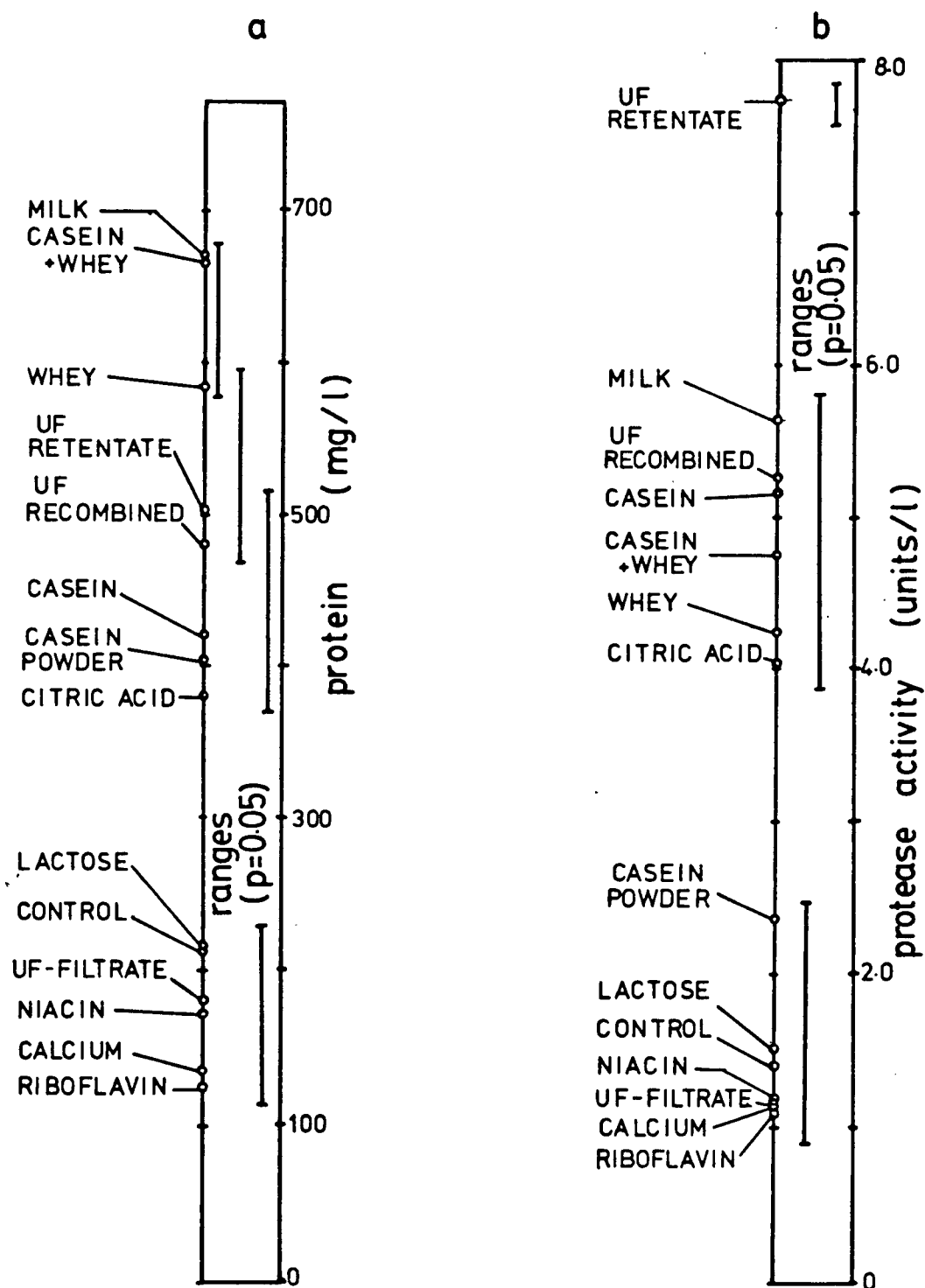


Figure 21. Effect of skimmed milk components on (a) protein, and (b) protease activity, in fig cell suspension cultures. UF refers to ultrafiltration products, retentate, filtrate, or these recombined (1:1). Range spans encompass groups of media producing significantly different results (Duncan's multiple range test, $p=0.05$).

Influencing synthesis and release of protein and protease: Table VIII includes data from two experiments to influence protein and protease productivity by materials not directly related to cell nutrition. The sulfur-containing compounds, glutathione and thiourea, were compared to B5 medium alone. Glutathione produced a 40% higher biomass, but protein and protease levels were not improved. Thiourea resulted in a decrease in all three factors (biomass, protein and protease), not a growth stimulant as suggested by Erez (1978).

Chloramphenicol and cycloheximide both had negative effects on biomass, protein content and protease activity in fig cell cultures. Chloramphenicol resulted in 83% of the cell harvest weight obtained in the control, while cycloheximide produced only 32% as much biomass. The drastic effect of cycloheximide is attributed to its mechanism of action, interference with the role of t-RNA in peptide bond formation at the 80S ribosomal subunit. Chloramphenicol also interferes with protein synthesis, but only on mitochondrial ribosomes. There was no significant difference in medium protease levels among samples.

The presence of the detergent Span-80, did not stimulate production of proteins in general, and protease activity was somewhat lower in this sample than in the control, 3.5 and 3.9 units/l respectively. Within each test set, biomass was paralleled by both protein synthesis and proteolytic activity.

It did not appear that proteolytic activity was due to rupture of dying cells, releasing intracellular (eg., vacuolar) proteases (Boller & Kende, 1979). Cells exposed to cycloheximide would have been expected to suffer damage, yet did not indicate higher enzyme levels than the control.

Table VIII Effect of miscellaneous organic materials on growth of fig cells in suspension culture, and production of protein and protease. Data from two different experiments are presented: (a) based on B5 medium, and (b) based on B5 with 3% skimmed milk. See text for concentrations of test materials. Relative values are based on the control. n= number of determinations of biomass, protein, protease.
s= standard deviation of the mean

medium		biomass (g/l)	relative	protein (mg/l)	relative	protease activity (units/l)	relative
(a)	B5	0.86 n=4, s=0.07	1.00	39.3 n=2	1.00	1.12 n=2	1.00
	B5 + thiourea	0.75 n=4, s=0.10	0.87	22.2 n=2	0.57	0.54 n=2	0.48
	B5 + glutathione	1.20 n=2	1.39	27.0 n=2	0.69	0.94 n=2	0.83
(b)	B5-M	6.47 n=3, s=0.41	1.00	407.9 n=3, s=6.57	1.00	3.91 n=3, s=0.12	1.00
	B5-M + chloramphenicol	4.37 n=3, s=1.02	0.68	185.4 n=2	0.45	3.24 n=2	0.83
	B5-M + cycloheximide	1.80 n=3, s=0.31	0.28	162.2 n=3, s=3.63	0.40	1.60 n=3, s=0.30	0.41
	B5-M + Span-80	7.33 n=3, s=1.85	1.13	425.8 n=3, s=5.35	1.04	3.507 n=3, s=0.18	0.90

Furthermore, fig cells grown in B5 plus skimmed milk generally gave evidence of casein hydrolysis, by clearing of the medium, within a week of subculture. Occasionally, clearing could be detected in four days. This activity, then, occurred well before mid-log phase and was not likely to be associated with mass cell death. As shown in Figure 19, the level of proteolytic activity detected decreased after the third week, during the stationary phase, when cell death became an important factor in the population.

A comparison of results of four experiments involving assessment of protease activity in fig cells is given in Figure 22 (a&b). The enzyme activities are presented in their two component fractions, in the medium (extracellular) and in the cell extracts (intracellular). With or without the addition of milk to B5 medium, a much larger proportion of total protein was contained in the cells than in the medium: average proportions of 0.95 and 0.80 in cells grown in B5 and B5-M, respectively. Protease activity was divided almost equally between cells and medium, average proportions in cells being 0.44 and 0.49 in B5 and B5-M media, respectively. However, there was a wide scatter in the distribution of enzyme activity, the proportion in cells ranging from about 0.3 to 0.7, so it was not possible to assay only one of these two fractions then multiply by a proportionality constant. The inclusion of the above test compounds in media did not appear to influence the proportion of extracellular enzyme activity.

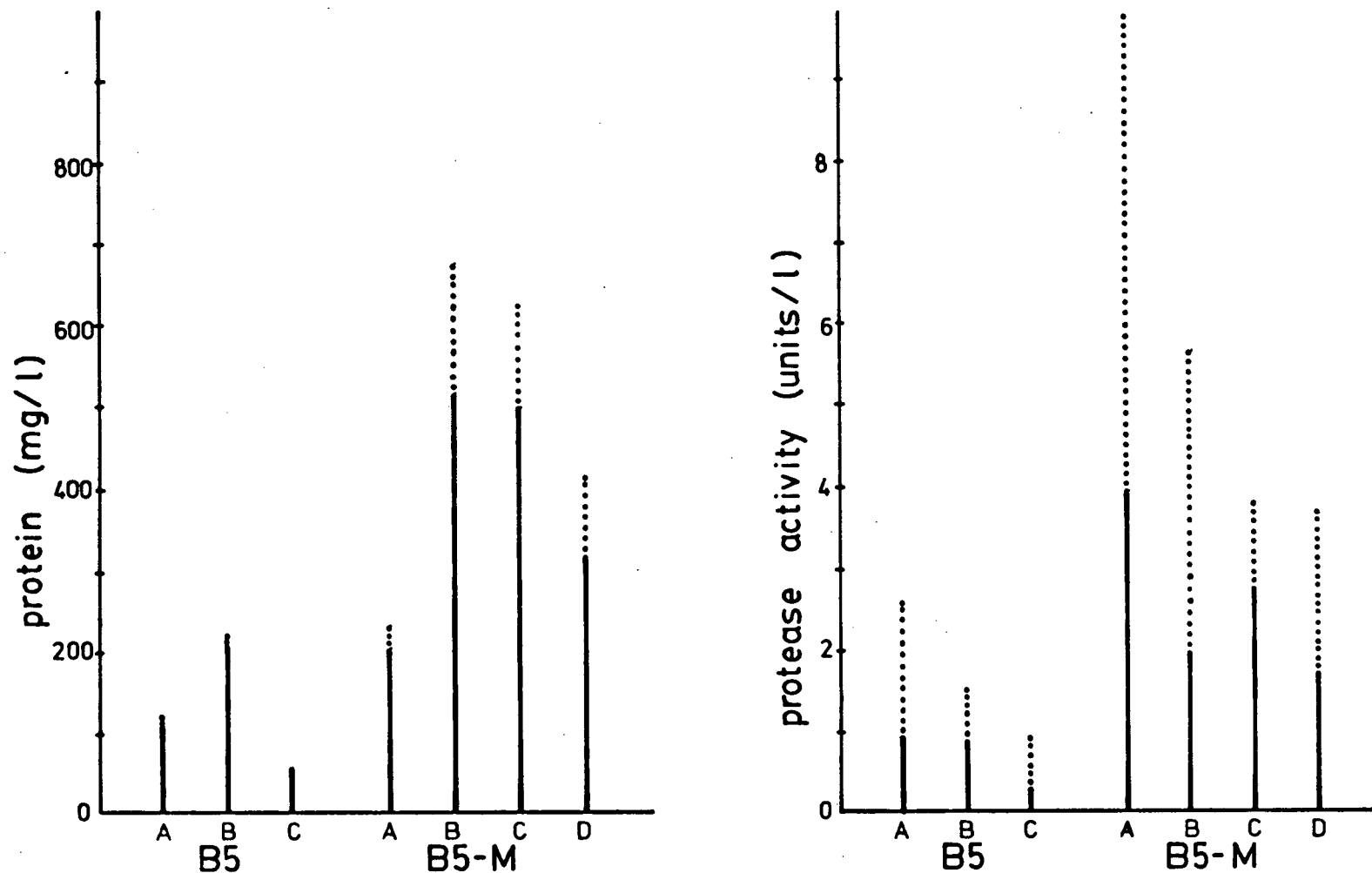


Figure 22. Intra- or extracellular location of protein and protease activity in fig cell suspension cultures. Both media and cell extracts were assayed. Data from three B5-grown cultures (A,B,C) and four B5-M-grown cultures (A,B,C,D) are presented, where the letter codes refer only to different experiments. Solid line, cell extract; dotted line, medium.

V. DISCUSSION

1. Tissue dedifferentiation

Successful utilization of plant tissue and cell cultures is dependent upon means of influencing cell growth and development. Callus cultures consist of immature and relatively unspecialized cells derived largely from cambial and parenchymatous cells. Propagation of such tissues is, of necessity, very labour intensive. Furthermore, tissues grown on semi-solid media are subject to varying degrees of morphogenesis due, in part, to the spatial association of cells with each other and with the medium. The desirable goal is a system in which all cells are maintained in identical conditions, preferably without fluctuation over time, such as continuous-feed fermentors. This situation requires a knowledge of cell growth, genetic status and metabolism, much of which has been derived, to date, from batch culture systems.

Several plant species were selected for the purpose of producing proteolytically active cell suspension cultures. These were Carica papaya, Ficus carica, Ananas comosus, Dieffenbachia amoena, D. picta, Galium verum, Cynara cardunculus and Cirsium arvense. Hundreds of plant species have been propagated as callus for a variety of purposes. Those that have not, either have not been investigated, or have particular environmental or nutritional requirements that have not yet been identified or accommodated. The species selected for this study were handled by the traditional methods of explant excision and incubation on two of the most common tissue culture media in use today, MS (Murashige & Skoog, 1962) and B5 (Gamborg et al., 1968). Developing calli were incubated under a single environmental regime: 28°C in total darkness. Over the first four to five months of establishing

cultures, only a few variables were exercised. These were the plant auxins (IAA, NAA, 2,4-D, 2,4,5-T, and p-cpa), transfer time (at callus initiation, after 2 weeks, after 5 weeks), inoculum size (2-15 mm in diameter), and medium supplements (yeast extract, casein hydrolysate, and skimmed milk). Visual examination of growing tissues determined the most suitable conditions for those species which did callus and develop in vitro.

Callus cultures were obtained from papaya, fig, dumbcane, cardoon and thistle. The first two of these showed the fastest growth. Explants from pineapple, dumbcane and mature cardoon were all subject to heavy endogenous contamination, and no pineapple explants survived more than three weeks. Bedstraw presented an unusual reaction, in that seeds developed no callus but stem explants resulted in a non-callus outgrowth to produce a root-like mass.

Fig and papaya tissues responded equally well to MS and B5 media. Of the auxins, IAA alone was less effective than the remainder, but could be used in combination with the phenoxyacetic acids, so p-cpa was arbitrarily adopted. The best transfer interval varied from one callus to another, but appeared to be suitable about one week after rapid outgrowth of friable callus. This generally resulted in transfers after 20-30 days, with the minimum fragment transferred successfully being about 5 mm in diameter. Both yeast extract and casein hydrolysate inhibited callus development, the latter resulting in browning of tissues. Skimmed milk was an effective growth stimulant. This supplement had been in use previously as an indicator of proteolytic activity in papaya callus cultures (Townesley, unpublished). Thus, skimmed milk in agar media provided an effective rapid method for determination of proteolytic enzyme production.

Callus initiation from papaya explants has also been reported from other laboratories (Medora et al., 1973; Arora & Singh, 1978; Litz & Conover, 1978; Medhi & Hogan, 1976). Plant propagation was the goal of Litz and Conover, while Medora's group reported the presence of proteases in their papaya callus cultures (Medora et al., 1973; Bilderback et al., 1976; Mell et al., 1979). Pineapple has also been used for micro-propagation, but this work bypasses callus formation by use of apical and axillary buds, thus requiring only a continuation of the natural morphogenetic sequence (Mathews et al., 1976). Development of callus cultures from fig has not been previously reported.

2. Cell suspension cultures

For economic reasons, the establishment of cell suspension cultures is really the first step towards large-scale production of plant cells or their metabolites (Dougall, 1980). The advantages of suspension culture systems include: faster growth rate, uniformity of cell environment and inoculum, simplicity of microscopic examination, ready alteration of the medium and the possibility of direct plating for cloning purposes (Widholm, 1980).

Fragments of the root-like outgrowth from bedstraw explants transferred to liquid medium continued to develop in the same differentiated form, resulting in large tangled masses. Microscopic examination showed no tendency to callus formation and little root-cap cell sloughing. Transfer of medium containing only free cells resulted in no further growth, so investigation of these cultures was discontinued.

Callus cultures of fig, papaya and thistle grew well enough to attempt suspension culture. Calli were dissected and transferred to liquid

medium of the same composition and maintained on rotary shakers, again at 28°C in total darkness. Because thistle grew poorly, even in milk-containing medium, suspension culture of this species was not pursued further. Papaya callus broke up slowly, forming a fine cell suspension by the third transfer, while fig cells readily dispersed within two weeks of introduction to liquid medium. Neither species presented the common problem of cell aggregation. Microscopic examination revealed single cells and small clumps up to about 20-30 cells in suspension cultures beyond the third transfer.

When skimmed milk was present in the medium, clearing of the milk turbidity could be detected in 5-10 days and thick cell slurries were formed in 14-20 days. It was apparent that Gamborg's B5 medium, particularly with the addition of skimmed milk (at a rate of 3%, v/v), was nutritionally adequate for growth. In terms of dry weight, a 50-65% conversion of sucrose was not unusual over a two-week period. Papaya and fig cell suspension cultures have not previously been reported in the literature, and the rapid, luxuriant growth attained by both was remarkable.

3. Determination of biomass, protein and protease

Cell harvest yield is the most common method of assessing growth in plant cell cultures (Rose & Martin, 1974). If dry weights are determined, this is also the most accurate measure available. Settled cell volumes and fresh weight yields have also been used as growth indicators (Nickell & Maretzki, 1969; Byrne & Koch 1962). Because water content of harvested cells is difficult to standardize, fresh weight values can be highly variable. Settled cell volumes from fig cultures were found to accurately reflect dry weight during the early phases of growth, but the latter method was selected for its reliability and generalizability. Papaya

and fig cells were found to be very different in size and structure, so it was supposed that they would pack differently. Gamborg et al. (1968) used cells dried 18-20 h in a vacuum oven at 60°C, as others have since. Kato and Asakura (1981) are among those who have used lyophilized cells. Due to the possibility of heat labile proteases and the potential for endogenous proteolysis, all harvested cells were freeze-dried to obtain biomass data prior to extraction. These weights bore a constant relationship to oven-dried weights from both papaya and fig cell suspensions.

Proteins are considered primary metabolites of plant cells in culture and their production is thus more closely linked to general nutritional status and rapid growth than are products of secondary metabolism. Plant cells in situ contain approximately 5% protein (db) in non-storage organs (Thomas & Davey, 1975). In seeds and tubers, protein content is more commonly 10-20% of the dry weight. Suspension-cultured fig and papaya cells grown in B5 medium with or without milk were generally found to contain 3.5-7.0% protein ($\bar{x}=4.55$, $n=45$) in crude extracts. This range is comparable to that reported by Gamborg and Finlayson (1969), a range of 1.4-8.7% protein in fourteen species cultured in vitro.

If Kjeldahl nitrogen values obtained for fig cells were representative, averaging 3.58% of the dry weight, then it must be surmised that approximately 80% of the nitrogen is either non-extractable or non-proteinaceous. It could be bound in the cell wall and membranes, or present as nucleic acids or other non-protein nitrogen (NPN) in the extracts. High absorbance values of unincubated TCA-soluble reaction mixtures in protease assays were an indication of high NPN content of cell extracts. This possibility was not considered in calculation of protein from Kjeldahl nitrogen by Gamborg and Finlayson (1969). The range of nitrogen contents

in fig cells was not particularly high in comparison to other literature values, lower than the reported 5-7% in tobacco cells (Kato & Asakura, 1981) and 8.3% in rice cells (Cifferi et al., 1980). The latter authors reported that suspension-cultured rice cells contain 46% protein (db), a very high figure in comparison to fig cells and even to rice grain, which is 6-8% protein.

Several methods of protein assay were considered for routine use in this project. The method of choice was the dye-binding technique of Bradford (1976). It was found to be sufficiently reproducible and sensitive for this work, but is especially to be praised for its simplicity and rapidity.

Most work detailing enzyme synthesis and activity in cell suspension cultures has dealt with the enzymes involved in normal metabolic functions, and little information exists with respect to enzymes with potential commercial application. Reports of proteases from papaya callus tissues in vitro are the exception, arising from a group of researchers in the United States--D. Bilderback, D.E. Bilderback, R. Medora, G.P. Mell, J. Ong and J.M. Campbell. It is mainly their work which stimulated the present study. They produced papaya callus cultures, then lyophilized and extracted these to test for protease activity on a variety of substrates. The authors used the assay method for papain given in the Food Chemicals Codex (National Academy of Sciences, 1966). Unfortunately, results were reported in absorbance units, and thus could not readily be compared herein.

The present work began with an assessment of alternative protease detection methods. Adapting the gelatin digestion method of Glenister and Becker (1961) to plant cell extracts provided the first evidence that proteolytic activity visualized by the clearing of milk in liquid media was

extractable and sufficiently stable for exogenous assay. This method, however, is not precise enough to be useful in comparison of cell cultures grown in media with only slight modifications. Three agar diffusion methods (Araki & Abe, 1980; Holmes & Ernstrom, 1973; "Protease detection kit" from Bio-Rad) all failed to detect activity that could be quantitated by the Food Chemicals Codex method. This is believed to be due to the failure of the enzyme(s) to pass through the agar medium, thus no zones of clearing, turbidity or colour change could be seen at any distance from the origin.

Fluorescence detection methods for proteases have received much attention in recent years because of their high sensitivity (Udenfriend et al., 1972; Schwabe, 1973). The technique reported by Spencer and Spencer (1974) relies on the loss of fluorescence as the substrate-bound ANS reagent is released upon proteolysis. Following the rapid initial increase in fluorescence upon addition of the plant cell extract, no significant decline could be seen over 30 minutes. It was postulated that the cysteine activator, or other substances present in the extracts caused an increase in turbidity of the reaction mixture due to aggregation of the casein substrate. The control reaction, using chymotrypsin, demonstrated the predicted loss of fluorescence. Another complication which arose was the operating temperature of the assays. For short-time incubation, it was clear that a temperature higher than ambient would be necessary. Another method, described by Chism et al. (1979), circumvented this problem by preincubation of the substrate-enzyme mixture, followed by fluorescamine labelling of TCA-soluble peptides. This approach appeared to work in the range of 50-500 ug tyrosine and with papain concentrations at the higher end of the range previously employed (200 ug per reaction tube). With the

amount of time, labour and cost involved, this method held no advantage over the Food Chemicals Codex assay.

A few modifications were made to the Food Chemicals Codex method for papain for ease of preparation and quantitation. The activator used, dithiothreitol rather than cysteine, was added at the start of the incubation period instead of being included in the extraction buffer. This simplified advance preparations and assured a minimum of proteolytic activity in the extracts prior to determination of protein content. Secondly, because activity was very low, it was convenient to decrease the substrate concentration from 10 to 2 mg/ml, a step which improved the precision of absorbance readings, possibly by improving the efficiency of casein digestion. Proteolysis in extracts of lyophilized cells was low, so the 0.2% casein substrate would rarely have approached complete hydrolysis. The incubation period was also extended to two hours, from one, increasing the differences between test and control samples. This would have been done more effectively by increasing the incubation temperature to 47°C. Unfortunately, at the time that temperature stability of the protease was determined, a great deal of data had been collected at 40°C, as recommended in the original method. This would not have been truly comparable to results obtained at a higher incubation temperature. Despite the lengthy and complicated procedure, this method was adopted for routine assay. It was, in part, compensated for by the simplicity of protein determinations done concurrently. A similar protease assay method involved addition of a ninhydrin reagent to the TCA supernatants for absorbance readings at 570 nm (Reimerdes & Klostermeyer, 1976). Besides being more cumbersome in that yet another reagent would be required and all supernatants would have to be pH-adjusted, there was interference by the enzyme activators such as cysteine or dithiothreitol.

Milk clotting activity has sometimes been mistakenly understood as an indicator of proteolytic activity (Pozsar-Hajnal et al. 1974; Pozsar-Hajnal & Hegedűs, 1975). Although commonly present in protease preparations, milk clotting activity is not necessarily directly correlated with proteolytic activity, as demonstrated by Skelton (1971) using papain. This distinction may occur due to a difference in optimum conditions for the two activities, and it could be possible to favour one or the other by manipulation of incubation conditions. Whitaker (1959) reported on the effects on pH, temperature, substrate concentration and inhibitors on the milk clotting activity of ficin, following similar methodology to that devised by Balls and Hoover (1937) for papain. To date, however, few plant species have provided sufficient milk clotting activity for the manufacture of cheese without also having too much proteolytic activity. Extracts of papaya and fig cell cultures yielded clotting times far in excess of a reasonable range for cheese manufacture. An investigation of conditions conducive to milk clotting may yet improve the potential for fig cell cultures in this field. A long clotting time may be desirable if conditions preventing formation of bitter peptides are defined. It was unfortunate that cardoon tissues could not be readily propagated in suspension culture, since this is one of two plants that have been used for traditional cheese-making (Vieira de Sa & Barbosa, 1970a&b). Insufficient young material was available for extraction to test milk clotting activity.

Proteolytic activity in fig and papaya suspension cultures became evident in 5 days at the earliest, by a loss of opacity in milk-containing media. Protease determinations over time established 14 days as the optimum time for productivity, hence, for harvest. In media without milk, this was

slightly delayed in papaya cultures, to 16-18 days, but unchanged in fig cultures. Location of enzyme activity, assessed only with fig cell cultures, showed no significant difference between B5 and B5-M with respect to the proportions of intra- and extracellular activity. It was clear that the entire culture (cells and medium) would have to be lyophilized, or otherwise concentrated, in order to obtain the full proteolytic activity. One solution might be achieved by altering the cell permeability, as suggested by Brodelius and Mosbach (1982) and Reese and Maguire (1969). Only one material, the detergent Span-80, was tested in this regard. No significant difference in medium protease activity was detected between Span-grown and milk-grown fig cells. The possibility still exists that a reversal of this principle might be applied, ie., addition to media of substances which prevent cell leakage of enzymes, thereby necessitating only the harvest of cells and requiring much less effort to concentrate. There appear to be no reports in the literature testing this hypothesis.

Assay difficulties: Several potential problem areas may be encountered in dealing with crude extracts of dried plant cells and in transforming yields of small volume cultures to large volumes. In this study, harvest weights were determined by collection of cells on Miracloth then lyophilizing the mass removed by scraping of the filter. Depending upon the total harvest weight obtained, the cells adsorbed into the filter may sometimes have constituted a significant proportion of the total weight. By estimation from pre-weighed filters, this loss was unlikely to be more than 5%. Also with respect to weights, lyophilization was chosen in order to better preserve endogenous protein and protease activity, although other

methods of drying yielded harvest weights about 10% lower (papaya) and 6% lower (fig). Where calculations based on biomass were made, the lyophilized weight was used. However, most data are reported on the basis of culture volume. This was done partly to avoid estimation of true dry weight from lyophilized weight, but mainly to provide a clearer picture of overall productive capacity with a view to fermentor-scale production.

Extraction of dried material was performed as rapidly as possible in the cold to prevent loss of either proteins or proteases due to proteolysis. The thoroughness of the adopted extraction procedure was not investigated, however, and it is possible that this may have varied from one sample to another. In compensation, a grinding time of 60-75 seconds using a buffer:dry weight ratio of about 15:1 became standard procedure.

The presence of endogenous protease inhibitors in cell cultures was a real possibility. Such compounds could have been active during protease assays. Materials shown to inhibit activity of standard ficin and/or papain preparations included trypsin inhibitor, calcium thiocyanate, sodium tetrathionate and thiourea. Sodium tetrathionate is a known inhibitor of sulfhydryl enzymes, and was included as a control: both papain and ficin were strongly inhibited in its presence, as expected. The inhibitory effect of trypsin inhibitor was somewhat surprising since the activity of plant proteases is not generally decreased by this material (Fossum & Whitaker, 1968).

It is believed that most plants probably contain protease inhibitors of some sort as a mechanism for regulation of metabolic enzymes associated with particular developmental periods, such as seed germination (Ryan, 1973). In fact, papaya contains endogenous isothiocyanates, shown herein to be inhibitory to activity of both papain and ficin. These have

not yet reported been in fig (Tang, 1974; Tang & Tang, 1976). The same mechanism may be responsible for the strong inhibition of papain and ficin reported here. Cyanide is known to be an activator of both enzymes (Arnon, 1970; Liener & Friedenson, 1970), but the thiocyanate form is inhibitory to their action. Ascorbic acid is present in papaya at the 1% level, and may also be produced by cells in vitro. Skelton (1968) demonstrated the inhibition of papain activity in the presence of 2.3 mM ascorbic acid, but without an enzyme activator. Because of this report and one by Whitaker (1959) ascorbic acid was not tested for possible stimulatory effects on standard enzymes or extracts. Lastly, thiourea was included both because it is a sulfur-containing compound, and because of a report by Erez (1978) that it stimulates growth of plant tissue in culture. Thiourea was included in liquid media in early attempts to establish cell suspension cultures for this reason, but because it appeared to produce no improvement in growth, it was subsequently omitted. If it could stimulate enzyme production, however, it may have become a useful addition despite having no effect on growth per se. This test showed inhibition of papain by thiourea (ficin was not tested), and thus dispensed with such a theory. In conclusion, the most effective enzyme activators were those used in other reports, cysteine and dithiothreitol.

Fig cell extracts indicated little effect of either sodium tetrathionate, trypsin inhibitor or calcium thiocyanate, with thiourea being slightly inhibitory. This leaves room for the possibility that proteases are already functioning under inhibitory conditions. Dithiothreitol was a stronger activator of proteases in the fig cell extract than was cysteine. If natural protease inhibitors were present in the cultures and extracts, their effects were at least partially overcome by these two activators.

Sodium diethyl dithiocarbamate showed an apparent stimulatory effect, but this can be attributed to an artefactual colour change achieved upon heating with the cell extract. The fact that these reactions do not entirely agree with those of standard ficin suggests that proteolytic activity of fig cell extracts is due, at least in part, to proteases other than ficin.

Protease assays were performed using Hammarsten casein as the substrate. No other substrates were used on a routine basis, though gelatin digestion was demonstrated. Proteolytic enzymes in plants often appear to be associated with protein turnover (Ryan, 1973). For this reason, they may be specific to the type of storage protein present in cells, such as the globulin-specific protease in pumpkin seeds (Spencer & Spencer, 1974). Although this specificity does not seem to apply to the ficin and papain extracted from fruit latex for commercial use, it may play a role in proteolytic activity detected in plant cell suspension cultures. The proteases present may have shown higher activity for more suitable substrates. However, it can be argued that only protein substrates of commercial importance should be used for assay if the eventual application is intended for this purpose.

No progress was made in identification of proteases from cell cultures. Electrophoretic separation of proteins in crude plant cell extracts should have made an important contribution toward answering questions of identity and multiplicity of proteolytic enzymes. It was assumed that proteases synthesized in cell culture would be present in larger quantity than the structural proteins and normal metabolic enzymes in cells. Although it was possible to visualize protein bands in a "pure" ficin preparation, almost no protein mobility was evident in crude fig cell extracts, even when concentrated as much as four times. Mercaptoethanol and

SDS treatments did not significantly improve mobility. There are at least two possible explanations for the lack of success. There may have been material present in the extracts which bound proteins, preventing their migration into the acrylamide gel, possibly the same forces prohibiting use of gel diffusion methods of protease detection. Alternatively, extracts containing 0.3-1.2 mg/ml total protein, as determined by Bradford's method, may not contain sufficient quantities of any particular protein to be visible as a banding pattern using the same stain.

4. Nitrogen nutrition in fig cell cultures

It was postulated, on the basis of dramatically improved growth in the presence of 3% skimmed milk, that proteins or other nitrogenous compounds were responsible for increased productivity of suspension-cultured fig and papaya cells. The approach taken to medium nitrogen supplementation was the addition of amino acids or other nitrogenous materials to the basal B5 medium. Growth was the primary indicator of suitability of the supplements. Production of protein and protease were also monitored, since growth alone could not ensure enzyme synthesis.

There is a large body of information in the literature on the subject of nitrogen nutrition and metabolism in plant cell cultures, as well as in intact plants (Dougall, 1977, 1980; Hewitt & Cutting, 1977). It has been estimated that 50-90% of total plant cell nitrogen is assimilated from environmental sources, the actual proportion being affected by availability and ease of metabolism (Hewitt et al., 1977).

Preferential sources of inorganic nitrogen vary among species, and nitrogen metabolism reflects the available sources (Tischner & Lorenzen, 1980). Reports indicate that the majority of plant cell cultures utilize

ammonium nitrogen before reduction of nitrate (Gamborg & Shyluk, 1970; Bayley et al., 1972; Dougall, 1980). This appeared to be the case in both papaya and fig cell cultures as well, evident in the initial decline and subsequent rise in pH of the growth medium (Gamborg et al., 1968; Bayley et al., 1972). Gamborg pointed out, in the same publication, that ammonium ions at levels greater than 2mM depressed growth of soybean cells in suspension culture, the same maximum experienced in intact plants (Mifflin & Lea, 1976). Only one paper encountered recommended a higher ammonium level, 10mM, possibly related to his goal of inducing embryogenesis in carrot cell cultures (Wetherell & Dougall, 1976).

Ammonia is assimilated in intact plants via two pathways, depending upon its concentration in the environment: either by glutamine synthetase activity, forming glutamine from glutamate, and/or by glutamate synthase activity, forming glutamate from α -ketoglutarate (Rhodes et al., 1976; Koiwai & Noguchi, 1972). The ratio of their activities is dependent on ammonium availability, the former being most important at low ammonia concentrations, but the latter becoming increasingly active at higher levels of ammonia, possibly to prevent ammonia toxicity. Some plant cells in suspension culture, such as wheat, appear to have no specific requirement for ammonia, while soybean cells are an example of a species which grows very poorly without it (Bayley et al., 1972). Gamborg and Shyluk (1970) reported that soybean cells would grow quite well on ammonium salts as the sole nitrogen source if Krebs cycle acids were also supplied.

The medium used herein, B5, contained 25 mM nitrate in addition to 2 mM ammonium ions [1 mM $(\text{NH}_4)_2\text{SO}_4$]. Ojima and Ohira (1978) stated that residual nitrate is usually sufficient for growth even after all the carbohydrate has been metabolized, although they also recommend 40 mM

nitrate to enhance growth of suspension-cultured rice cells. Utilization of nitrate requires activation of nitrate reductase, which in turn requires adequate medium molybdenum and calcium, and quite possibly the induction of a nitrate-specific permease (Oaks, 1977). Nitrate may be reduced to nitrite in the cytosol and reduced further, to ammonia, most likely in plastids such as chloroplasts (Mifflin & Lea, 1976). The potassium nitrate supplied in B5 medium is probably used to produce both nitrite and potassium malate (DeKock et al., 1977). If amino acids are to be synthesized from nitrate, both ATP and NADPH are required. This energy and reducing power, Fowler has suggested (1978), could be derived from the pentose phosphate pathway of carbohydrate metabolism and would therefore be unavailable under conditions carbohydrate deficiency. In the intact plant, nitrate need not be processed immediately, but can be transported through the xylem or stored in vacuoles without prior reduction (Oaks, 1977). Storage in intracellular pools may occur in single cell culture as well. The reduction products, ammonia and amino acids, and the cytoplasmic nitrate pool all serve to inhibit further nitrate uptake. Inadequate carbohydrate supplies accomplish the same effect (Bidwell et al., 1964).

Effect on biomass: It was evident, from the growth of fig cells in B5 medium lacking ammonium, that nitrate was an adequate nitrogen source. On the contrary, ammonia was insufficient as the sole nitrogen supply, not a surprising revelation due to the low concentration in comparison to nitrate. What was unexpected, though, was the inability of individual amino acid supplements, and even milk, to compensate for the nitrogen shortage. Apparently, the sum of nitrogen present in the medium is closely linked to growth, but not in a linear manner. Fig cell growth in the total of 7 mM nitrogen supplied by ammonium sulfate plus glutamate, for example, exceeded

half the yield from medium containing 25 mM nitrogen in the form of nitrate alone. Similarly, the combination of ammonia and nitrate nitrogen in B5 medium produced a higher biomass yield than the sum of the individual yields. The nitrate concentration in B5 is not excessive for fig cell suspension cultures, since a one-half reduction in this nutrient resulted in a concomitant decline in yield. Efficient nitrate utilization, however, seemed to have required the presence of a reduced source of nitrogen, either ammonia or some of the amino acids.

Amino acid metabolism in suspension-cultured plant cells has been reviewed by Dougall (1980) and Ojima and Ohira (1978). Amino acids are a reduced form of nitrogen but cannot entirely replace ammonium salts. Glutamine-grown soybean cells were found to produce only about two-thirds the yield obtained in the presence of ammonia, for example (Gamborg et al., 1968). The inclusion of amino acids in plant tissue culture media has wide-ranging effects on growth from strongly inhibitory to strongly stimulatory, depending on the plant species, inorganic nitrogen nutrition, nutritional history of the culture and the particular amino acids tested (Maretzki & Thom, 1978; Mifflin et al., 1977). Following are a few examples: (a) rice callus grew best when supplied with any of alanine, arginine, asparagine, glutamic acid or proline (Furuhashi & Yatazawa, 1970); (b) soybean root cells required the combination of lysine, arginine, histidine, aspartic and glutamic acids or protein hydrolysate for best yields (Gamborg et al., 1968); (c) the most useful amino acids for growth of sugarcane cells were found to be arginine, histidine, aspartic and glutamic acids (Nickell & Maretzki, 1969); (d) alanine and aspartic acids stimulated growth of Datura innoxia cells while most other amino acids fed individually were inhibitory to growth (Fukunaga & King, 1982).

There are also at least two reports of inhibitory effects of some amino acids being reversed by others. Cattoir-Reynaerts et al. (1981) found that lysine and threonine inhibition of growth in barley cultures was reversed by the addition of arginine. Growth of tobacco, tomato, carrot and soybean cells in nitrate medium was inhibited by a common spectrum of amino acids, with strongest effects on each species being seen with different particular amino acid supplements. The same authors (Behrend & Mateles, 1975) reported the abolition of such inhibitory effects, most notably by the addition of arginine or isoleucine.

A selection of amino acids, based on these reports in the literature, were added individually to B5 medium deficient in either nitrate or ammonium. In the presence of nitrate, six amino acids stimulated growth above the unsupplemented level, with only glycine producing a decrease in biomass. Aspartic and glutamic acids resulted in yields more than double that achieved with ammonium, all in the presence of nitrate. This indicated a near complete utilization of the amino acid nitrogen, presented at 5 mM in place of ammonium sulfate nitrogen at 2 mM. By the same reasoning, cysteine was utilized at one-sixth, and alanine at about one-third the efficiency of ammonium assimilation. Because no combinations of amino acids were tested, antagonism to growth inhibition could not be investigated.

In nitrate-deficient media, fig cell growth was poor with or without supplementation by the test amino acids. However, the stimulatory effects of glutamic and aspartic acids were again noted. Biomass yields in ammonium media were all less than 1 g/l so it would not be realistic to calculate percentages of growth inhibition below the unsupplemented ammonium medium. Fig cells appear to require both nitrate and a source of reduced nitrogen for best growth. The latter was adequately supplied by ammonium or

the acidic amino acids.

While skimmed milk was stimulatory to growth in both nitrate and ammonium media, it produced a greater biomass yield in the former. No other supplement to B5 medium tested resulted in equivalent stimulation of growth. Improved nitrogen nutrition may, however, induce protein synthesis, potentially enhancing production of proteolytic enzymes. Cell yields alone could not provide this information for fig cell cultures.

Effect on protein and protease: The protein contents and protease activities of cells and media were determined and compared with respect to the nitrogen sources provided. Two series of supplements were studied--the amino acids discussed above, and a few plant and animal proteins. Skimmed milk was included in both series for comparison.

Of the amino acids used to supplement B5, aspartic and glutamic acids induced the greatest protein production in both nitrate and ammonium media, though the latter, as a group, gave much lower protein yields. In nitrate media, arginine, alanine and cysteine were also stimulatory of protein production, relative to the basal levels. The same five amino acids also stimulated protease activity in nitrate media, while the other amino acids were inhibitory. Protease activity in ammonium-based media, regardless of supplement, was consistently lower than in the unsupplemented medium. No amino acids induced significantly higher proteolytic activity than unsupplemented ammonium medium.

These results reiterated the ineffectiveness of ammonium-based media when enhancement of protease production was the goal. The stimulatory effect of aspartic and glutamic acids was most likely related to their ease of assimilation and metabolism in protein synthesis. These compounds supply reduced nitrogen, carbon skeletons for carbohydrate and hydrocarbon

metabolism, and available fuel for ATP production.

Some of the proteins added to B5 medium interfered with determinations of harvest weights due to insolubility, undigested protein becoming trapped with cells upon filtration. Similarly, this residual protein resulted in artefactual cell protein levels. For this reason, only data for protease activity was evaluated for the purpose of medium improvement. This data indicated that many proteins, including reagent grade casein powder and enzymatic casein hydrolysate were inhibitory to proteolytic activity of cultures. The causes of their inhibitory action are not known, although casein hydrolysate had been shown, earlier, to be inhibitory to growth of callus tissues. Soluble starch, added only as a control for the effect of the presence of high molecular weight material, also proved to be inhibitory. Only fresh skimmed milk and egg albumen showed significant stimulation of proteolysis, to approximately the same level. Note that egg albumen is not heat-stable, and thus coagulated upon autoclaving the medium. Much undigested albumen remained after the incubation period, yet its presence had stimulated proteolytic activity.

A comparison of the effects of skimmed milk in complete, nitrate-deficient and ammonium-deficient media exposed a very broad range of protein levels. All milk-containing media yielded highest protein yields, but ammonium medium supplemented with milk was not significantly better than all the media without milk. This result was supported by the protease determinations, which clearly placed the NH_4 -milk combination at the same level as nitrate alone. Without skimmed milk, complete B5 produced highest proteolytic activity, and B5 with ammonium alone yielded the lowest activity.

5. Stimulatory effects of skimmed milk and milk components

Inclusion of skimmed milk in B5 medium resulted in higher yields of biomass, protein and proteolytic activity than could be obtained with any other nitrogen-containing supplements. It was therefore postulated that the stimulatory effects observed were due to non-nitrogenous materials. The partial fractionation of fresh skimmed milk permitted an evaluation of the possible relationship of the active principle(s) to milk casein and whey. Both of these components enhanced growth, relative to unsupplemented medium, although whey appeared to be more effective than casein or intact milk. Lactose, calcium, niacin or riboflavin did not significantly alter biomass yields. The filtrate from ultrafiltered whey was also ineffective, while the retained fraction significantly stimulated growth of fig cells.

The stimulatory effect of citric acid on growth was striking. Citrate provides available carbon for metabolism via the tricarboxylic acid cycle, contributing to an improved energy pool for the synthetic pathways associated with growth. Its use in plant cell culture media is not well documented. Gamborg and Shyluk (1970) found that soybean cells could grow with ammonia as the sole nitrogen source if Kreb's (tricarboxylic acid) cycle intermediates were provided. The function to these acids in cell cultures was not known, but several options were proposed: relief of ammonium toxicity, enhancement of ammonium transport or satisfaction of a carbon requirement for amino acid synthesis. Ojima and Ohira (1978) also found that some of the carboxylic acids, in supplement to ammonia, promoted rice cell growth. One application of citric acid to plant cell culture medium which is of particular interest was reported by Erner et al. (1975). Citrus species, especially orange, were known to require approximately 10% orange juice in the growth medium. This group discovered that the

stimulatory effect of orange juice was completely reproducible with citric acid (2.5 g/l). Figs, the fresh fruit, contain 6 meq citric acid per 100 g fresh weight, but no information on citrate content of the vegetative plant was available. It is possible, then, that a high endogenous citrate level is critical to normal metabolic activity in fig cell. The capacity to synthesize the required levels may be lost over the course of successive cell transfers (Erner et al., 1975).

Protein productivity of fig cells in media containing citric acid or the milk fractions, whey or casein, was significantly higher than that in unsupplemented B5. As usual, total protein content closely followed biomass production, with calcium, lactose, and the vitamins resulting in protein levels not significantly different from B5 alone.

A similar pattern was observed in assessment of proteolytic activities of fig cell suspension cultures grown in basal medium containing milk or its components. Whey, whey retentate from ultrafiltration, fresh casein, milk and citric acid all induced proteolytic activities significantly higher than other media. Casein powder was not significantly stimulatory, whereas fresh casein was, suggesting that the active principle(s) was/were unlikely to be the casein itself. A complication arises with the hypothesis that citrate was responsible for stimulation of growth and of protease activity. The ultrafiltrate from whey, which should have contained low molecular weight materials (less than 10,000 daltons), including citrate, actually showed no growth or protease enhancing effects. The most effective milk fraction in these respects was the whey ultrafiltration retentate. This discrepancy may have arisen as a result of co-precipitation with calcium and phosphate at neutral pH. Citrate, soluble in whey during the process of casein coagulation, may have become insoluble

in neutralized whey and could not pass through the ultrafiltration membrane.

6. Applications, problems and potential of plant cell cultures

A brief look at both positive and negative aspects of plant cell culture can help to put the present work into perspective in terms of applicability. The introductory chapter presented an overview of the multiple research directions taken by those propagating plant tissues and cells. Yet much of this work is still in the research stages, or is intended solely for research purposes.

Growth of fig cells could be improved by the addition of whey to basal B5 medium. Under these conditions, it may be possible to omit or decrease the plant hormone levels. Technically, the resulting cell slurry could be considered the equivalent of the same species in differentiated form. As such, difficulties in meeting regulatory guidelines for FDA approval would be minimal. The spectrum of requirements and tests for GRAS status described by Nelson (1980) and Whitaker (1980) might be avoided altogether for papaya cell cultures, if the presence of papain can be proven, since papain has already been declared GRAS. Application of cell suspension cultures would require development, but it is not difficult to envision meat marinades or yogurt containing such a puree. Refrigeration would prevent activity before use of a marinade, or after setting of a milk-based product. In the latter case, the slow milk clotting activities encountered in papaya cell suspension cultures may actually be favourable in terms of shelf life. Recent advances in techniques of whole cell immobilization (Brodelius & Mosbach 1982), though potentially useful for plant cells producing flavour constituents, would not likely be applicable to these protease producers.

Application of plant tissue and cell culture techniques has met with a few major hurdles. Underlying many difficulties lies the influence of genetics, both of the parent plant and of progeny cells. Much information is available on cytogenetic studies but few generalizations have been substantiated. Plant cells are commonly held to be totipotent, to contain the genetic information required to regenerate new, fully-differentiated plants. Within the mature plant, expression of genetic information is limited by surrounding cells and regulated, in part, by hormones. Such limitations to genetic expression are believed to be fully reversible (Street, 1977). Undifferentiated cells growing in suspension culture should, therefore, respond only to environmental conditions, which are under external control. This hypothesis has been supported by the large numbers of plant species that have been propagated in vitro then responded to deliberate induction of morphogenesis (Reinert & Bajaj, 1977; Murashige, 1978; Winton, 1978). The fact that some species have not yet been successfully subjected to dedifferentiation followed by morphogenesis could be explained either by a lack of totipotency or by a lack of knowledge with respect to nutritional and environmental requirements for these transformations.

A corollary genetic phenomenon concerns metabolic variation among calli (Townesley, 1977) and cell suspensions (Street, 1977). Rather than the temporal cytological changes accompanying cell differentiation, metabolic differences may be manifest concurrently. That is, cells at the same age and under the same culture conditions do not necessarily exhibit identical metabolic behaviour. Street suggested that one cause may be the continuous production of genetic variation. This could very likely be the case in callus and batch suspension cultures, which are in a continual state of

flux, providing a selective advantage for different fractions of the cell populations over time. Kibler and Neumann (1980) found a wide range in ploidy levels in Datura and barley cultures. They described cells as falling into one of two genetic classes, meristematic or parenchymatous. Their appearance was distinguishable microscopically though this difference has probably been mistakenly attributed to cell age in other work. Differences in nucleic acid content may then be manifest metabolically, noted as quantitative differences in productivity.

To avoid cell heterogeneity, cell selection has often been recommended. The simplest approach involves repeated transfer of a small inoculum of rapidly-growing cells, those best suited to the culture conditions (Noguchi et al., 1977). This method was applied, in this study, to papaya and fig callus and suspension cultures. Although proteolytic activity varied from one generation to the next, the range of variation did not appear to exceed three- or four-fold. These cell populations were not entirely homogeneous so productivity represented the mean of all cells. It is quite likely, though not investigated herein, that the differences between individual cells were much greater, particularly in the earliest callus cultures.

Related to cell selection is another field requiring further investigation--rapid methods of cell identification. Tabata et al. (1978) selected for high-nicotine producers on the basis of examination of small calli. They had a rapid method to identify the compounds of interest, the "cell squash method" and paper chromatography for alkaloids (Ogino et al., 1978). They found the selected cultures to be quite stable in the level of nicotine production over successive transfers. No such method was available for detection of high protease producers. Timing the clearing of

milk agar showed no differences among calli. Noting pH changes by inclusion of indicators in agar was also unsuccessful in detecting differences. The Bio-Rad protease detection method was applied in the preliminary search for a rapid detection method, again without results. For lack of such a selection method, the approach chosen was that dependent on endogenous selection pressures, using only rapidly-growing tissue.

A more refined technique for cell selection, described by Bergmann (1977), is based on the microbiological method of plating in agar. Cell densities of 10^3 to 10^5 cells/ml are generally required to induce growth. For most plant species, there appears to be a minimum cell density, below which no growth occurs. Successful plating results in a dense cell population in agar, upwards of two clones per mm^2 not conducive to inclusion of indicators in the medium. Cell plating is technically a difficult procedure and has met with limited success. This was not attempted with papaya or fig cell suspension cultures.

Zenk (1978) classified plant cells in two groups in terms of productivity in vitro: (a) all cells having similar productive capacity, usually less than the parent plant, requiring that the entire cell population be influenced to increase productivity, or (b) productive capacities differing among cells in a population, with a few variants being much more productive, sometimes more than evident in situ. The variability noted among papaya and fig cultures suggested that either the variability within cell populations was relatively small, or that cells with high growth rates included variants with high proteolytic activity.

Cytogenetic instability is one of the major difficulties encountered by plant tissue culturists. It appears now that there are several solutions to this. Cell selection, as discussed above, is only one

answer. Others include methods of haploid cell production from ovules, anthers or microspores, or by chromosome elimination. Details of these techniques are provided in several chapters in Plant Cell, Tissue and Organ Culture, edited by Reinert and Bajaj (1977), and in Plant Cell Cultures: Results and Perspectives, edited by Sala and others (1980). Furthermore, desirable cells can sometimes be readily identified by characteristics such as pigment production, alkaloid content and nucleic acid content. Recent advances have also been made in the application of radio-immunoassays (Weiller, 1977), and in identification of genetically linked characteristics, "genetic markers" (see Sala et al., 1980).

With the aid of sensitive detection methods, cell selection is simplified, and genetic variability is minimized. Plant cell clones could, in theory, be tailored to specific purposes: production of certain metabolites, biotransformation of precursor materials (Alferman & Reinhard, 1980), resistance to disease or environmental stress (Vasil et al., 1980), autotrophy, or catabolism of xenobiotics. Plant species and purposes may be matched and modified, but each situation requires special consideration. The use of papaya or fig cell suspension cultures for the production of proteolytic enzymes is a real possibility. This work has provided only an introduction to this plant:purpose relationship. Improvement of productivity, and a clear definition of nutritional essentials, must precede attempts at bioreactor-scale production. Plant cell culture is an expensive proposition, worthwhile only when the cost of harvesting the original plant becomes prohibitive, or the plants become extinct. Horticulturists, plant breeders and plant pathologists are interested in plant tissue culture as a propagative method and model study system, as indicated by the August/82 special issue of "California Agriculture", an applications-oriented

publication of the University of California, Department of Agriculture.

With regard to plant product synthesis, however, only industries related to pharmaceuticals have looked seriously at plant cell culture as a means of production, despite the costs. A lack of knowledge and expertise in the industry have so far stymied developmental work. There is a requirement for long-term research and development programs due to the slow, labour-intensive establishment of cultures and cloning procedures. It would appear that the North American food industry does not yet have the need or incentive for such a commitment.

VI. SUMMARY

Tissue cultures were established from explants of cardoon (Cynara cardunculus), thistle (Cirsium arvense), dumbcane (Dieffenbachia amoena), papaya (Carica papaya), and fig (Ficus carica). Two species failed to form callus; pineapple (Ananas comosus) and ladies'bedstraw (Galium verum). Cultures were all maintained at 28 °C in darkness, preventing normal photosynthetic activity and differentiation.

Papaya and fig were successfully maintained under these conditions as undifferentiated cell suspensions. Growth in liquid media produced greatest cell dry weight in 14-21 days, the faster-growing cultures being selected for sub-culture.

Proteolytic activity was apparent in tissue and cell cultures by the clearing of milk in media within a week of sub-culture. Casein was selected as the substrate for quantitation of extractable protease activity. Assay results were used to compare stimulatory effects of medium supplements, and determine temporal productivity. The FCC method, in modified form, was used routinely with tyrosine content of the TCA-soluble components as the unit of measurement. Extractable protein was routinely determined using the dye-binding method of Bradford. Biomass, cell dry weight produced over the test period, was determined by freeze-drying samples. All data were converted to a per-litre basis for consistency and ease of comparison.

B5 medium, which contains 2% sucrose, was an adequate nutrient supply for growth. No modifications were made to the basal ingredients except where specified. Supplements to B5 medium were investigated for their influence on growth and production of protein and protease. Skimmed

milk, at 3% v/v, was one of many supplements. Both papaya and fig cell cultures produced proteolytic enzymes that would hydrolyse milk casein in the medium within one week. Other medium supplements included amino acids, thiols, proteins, starch, lactose and other milk components.

Results showed that protein synthesis and proteolytic activity in extracts of lyophilized fig cell cultures followed growth (biomass production) closely. Those medium supplements which stimulated cell growth, such as milk, led to higher levels of total protein and protease activity. Thus, nitrate in B5 medium was shown to be more important, in terms of overall growth, than ammonia as a source of inorganic nitrogen for fig cells. The most effective amino acid supplements for stimulation of growth and protease production in fig cell cultures were glutamic and aspartic acids, with cysteine also improving protease activity, but not growth. Of the proteins and peptides added to B5 medium, only milk and egg albumen produced significantly higher protease activity than the control. Dried casein and casein hydrolysate were both ineffective medium supplements. The cause of the stimulatory effect of egg albumen is not known.

Data obtained from fig cell suspension cultures grown in B5 supplemented with milk or various components thereof, showed that growth and protease activity were highest with whey, milk, fresh casein and citric acid. Unsupplemented medium resulted in significantly lower biomass, protein and protease levels. The beneficial effect of milk may be largely due to its citric acid content, which would have supplied growing fig cells with an additional energy source and carbon source. It could also contribute to intracellular levels of citrate, possibly driving cell metabolism at a rate greater than normal. Whether cells grown in this

medium were normal in size or ploidy was not determined. Since whey produced such a dramatic increase in biomass, although not quite as high as intact milk, this unrefined material could be used in liquid form as an inexpensive supplement for growth of fig cells. It would also be worthwhile investigating for propagation of other plant species in tissue or cell culture.

There was much variability among papaya and fig cell suspension cultures, often not stable from one generation to the next. Each experiment required a standard inoculum for each test condition, and cells grown in the same medium did not necessarily produce comparable results if the inoculum differed. It would have been desirable to select clones on the basis of high protease productivity, but no method for rapid and non-destructive identification was available. As a result, cell suspension cultures of papaya and fig probably contained fast-growing cells with a range of productive capacities, total data upon assay being representative of the average.

Plant cells propagated under controlled conditions such as present-day industrial fermentors or bioreactors, could become an excellent, reliable source of proteolytic enzymes for the food and other industries. This basic research indicates that both papaya and fig cells can be dark-grown as cell suspension cultures and will both demonstrate proteolytic activity.

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