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Optimized Growth Conditions and determination of the Catalytic Type of the Peptidase Complex From a Novel Callus Culture of Pineapple (*Ananas Comosus*)

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ABSTRACT

Callus tissue culture of pineapple [*Ananas comosus* (L.) Merr.] is here focused to obtain maximal proteolytic activity. Biochemical parameters, such as pH optimum, optimal temperature range, thermal stability and protein content of the enzyme preparation were assessed for the first time. Catalytic behaviour, substrate-specificity and the use of a variety of inhibitors revealed its cysteine proteinase nature, suggesting it must be called callus bromelain. Comparison of *in vivo* and *in vitro* bromelains is also discussed, the latter being in many respects similar to a purified commercial stem bromelain, further supported by standard and automated fast electrophoresis, and capillary zone electrophoresis. The proteolytic activity of the callus bromelain showed to be higher than that obtained from the plant.

Key Word Index- *Ananas comosus*; Bromeliaceae; monocotyledons; angiosperms; pineapple; "ananá"; callus culture; biochemical parameters; catalytic type of peptidase complex; callus bromelain.

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INTRODUCTION

Ananas comosus (L.) Merril [*Bromelia comosa* L.; family, Bromeliaceae; common name, "ananá", "pineapple"] is a herbaceous tropical plant native to northern Southamerica, which is cultivated in northern Argentina for commercial purposes due to its edible fruits [1]. The pineapple is one of the major fruit crops of all tropical countries. There are many named cultivars usually under the same english common name, pineapple, though only a few are prominent. "Smooth Cayenne" with non-spiny leaves is the most widely grown, "Red Spanish" in the Caribbean area, "Abacaxi" in Brazil, "Queen" in South Africa and Australia, "Singapore Spanish" in Malaya, "Cabezona" of Puerto Rico and "Sugar Loaf" of the Bahamas are large and very sweet [2]. Choice of cultivar, and to some extent, disease and pest control and resistance to herbicides affect the rate of growth and the fruit production. There is extensive literature on fertilizer trials and variations in agronomical parameters in order to improve fruit quality as well as total yield.

In Argentina is well known the so-called "ananá", the same as the "abacaxi" from southern Brazil, with spiny leaves and cylindrical fruits *ca* 25 cm long and 15 cm thick, yellow to brownish when ripe, which is very well distinguished from the so-called "piña" with round-oval

smaller greenish fruits. This "ananá" was studied in this paper. In fact, it is believed that the pineapple originated in Brazil, and that spanish and portuguese traders introduced the plant in India, China and Africa before the end of the 16th century, and in Hawaii at the beginning of the 19th century [2]. There is today a growing demand for the fresh and processed fruit and its juice, and for the bromelain for industrial purposes.

The enzyme complex of *A. comosus* called bromelain [3] is known for its clinical applications [4], particularly modulation of tumour growth, blood coagulation, third degree burns, improvement of antibiotic action and anti-inflammatory properties of therapeutic value [5], including treatment of post-chirurgic edems, inflammation of the respiratory tract, thrombophlebitis and venous ulcer [6,7]. The mechanism of action of the drug relates in part to its modulation of the arachidonate cascade [5]. Moreover, proteolytic enzymes such as bromelain inhibit the action of cholera toxin [8], and

are also choice enzymes for food processing [9]. *In vitro* and *in vivo* studies also revealed antineoplastic properties of bromelain [10].

Isolation procedures, physical properties, chemical and catalytic characteristics of stem and fruit bromelains (EC 3.4.22.32 and EC 3.4.22.33, respectively [11]), and the two additional ananain (EC 3.4.22.31) [12] and comosain [13] detected only in the stem, have been extensively reported and reviewed [14] as obtained from the pineapple plant, cation exchange resin and gel chromatography methods being the most often cited for isolation steps. Crude purified bromelain extracts were also obtained from fruit juice using sequential batch membrane processing systems [15]. Bromelain is also the commercially available dried powder usually prepared from waste pineapple stem material.

There have been considerable confusion around these enzymes and many contradictory reports have described up to six different components in the stem and at least two componentes in the fruit, possibly depending on the geographical location [14]. Although some authors have reported that up to six enzyme forms can be obtained from the fractionation of the stem bromelain, two forms seem to be the most abundant and well separated by means of high-resolution ionic exchange chromatography. These forms possess nearly identical secondary and tertiary structures according to their circular dichroism spectra [16].

A number of reports of bromelain are related to *in vivo* pineapple and micropropagation techniques from the agricultural point of view [17-20], *e.g.* a novel method for rapid micropropagation of two cultivars of pineapple based on shoot elongation induced *in vitro* [19]; culture conditions of *A. sativus* coupled with manipulations of the *in vitro* techniques and surgical treatments leading to the rapid multiplication of the pineapple plant [17], including histological studies. All of them using other culture media than those described herein and, in fact, performed under other objectives. Recently, tissue cultures and bromelains in callus tissues of *A. sativus* were reported by researchers from China [21], under other culture media conditions and only measuring the proteolytic activity, showing as in our case to be higher than that in the natural plant. Chemical analysis and enzyme properties/behaviour of tissue cultures were not reported in any of the earlier reports.

The present paper deals with the study of callus of *Ananas comosus*, searching for proteolytic activity, characterization of the catalytic type and optimization of the culture growth conditions. Callus cultures were obtained which accumulate proteinases of the bromelain-type, namely callus bromelain, in concentrations higher than those of the pineapple plant. We further examined the biochemical parameters and compared with each other the enzymic properties of the callus bromelain and the fresh pineapple plant bromelains.

As far as we know this is the first report on the *in vitro* characterization of the proteases' complex from callus of pineapple (*Ananas comosus*) obtained on selective media, their mechanism of action and the determination to which catalytic type it belongs.

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Undoubtedly, the *in vitro* culture is a source of interest for providing homogeneous material, and allowing the optimization of the production of target compounds.

RESULTS AND DISCUSSION

Since the last two decades, plant cell and tissue culture have been complementing the conventional methods in finding solutions to some of the problems in agriculture and forestry [22]. There are also a lot of applications in the study of the biosynthetic pathways, plant physiology and genetics. A very potential but not so widely explored application is the *in vitro* production of natural plant metabolites [22] to be used as pharmaceuticals, flavour, foods and many other fine chemicals including even the enzymes.

Proteolytic enzymes of papain-type seem to be widespread in Bromeliaceae [23, 24]. Papainases were found in stems of *Ananas comosus* var. *Cayenne* [25] and other members of Bromeliaceae, being the only monocolyledons which accumulate these enzymes. However, the various cysteine proteinases present in the plants of the Bromeliaceae seem to possess distinct properties [13].

Tissue from any plant part can be used to establish the initial cell culture, but that with major capacity of induction must be selected and put onto a suitable growth medium, where it starts dividing and forms a callus. This callus is excised from the donor tissue and maintained by replenishing its medium. By handling the medium, particularly the growth regulators it can be induced callus tissue to regenerate individual organs or a complete plant. This was done in this paper in order to get plant material for herbarium purposes.

Another feature of these selection methods is to stimulate uptake of nutrients from the medium in order to get high yields of the target-enzyme. A new culture medium which had been previously developed in our laboratories [26] allowed to increase the growth efficiency for obtaining "ananá" plantlets *in vitro*, growing more quickly and successfully than with a standard medium. These plantlets obtained from the axillary buds of the ripe pineapple crown were cultured in inorganic nutrients suitably supplemented to induce callus. The sterile axillary buds had nearly the highest induction ratio. The callus obtained was subcultured into fresh medium (see Experimental).

Different culture media were developed in our laboratories [26] by modification of vitamins and growth regulators, resulting in better friable calli with Murashige and Skoog (MS) vitamins and the auxins used, the best results being those obtained with α-naphthaleneacetic acid (ANA). Four (M1 to M4) of all these culture media were selected, which consisted of different compositions accomplished upon variation of the sort and quantity of the cytokinin (see Experimental). This selection exploits the fact that most plant tissue cultured *in vitro* require exogeneous cytokinin for optimal growth. Two of the most widely used cytokinins in plant tissue culture are benzyladenine and isopentenyladenine. Different concentrations of benzyladenine (BA), 1.0, 0.5, 0.0 and 0.0 ppm, were added to a constant concentration of ANA (10 ppm) throughout the media (M1 to M4) assays, while kynetin (K) was only used in M4 (0.5 ppm).

Pineapple plant bromelains are currently completely precipitated by ammonium sulfate, methanol, isopropanol and also acetone, the latter being the most suitable for large-scale extraction. Therefore, though the protein constituents of "ananá" callus could be concentrated by a variety of methods, acetone was used in the present study in order to compare materials obtained by the same procedure [14], and further worked-up as usually.

The proteolytic activity was evaluated primarily in a qualitatively way (Table 1) according to the caseinolytic activity evidenced around each callus in the Petri dish. The growth rate and friability of the calli were also measured in each culture media. Experiments were carried out with 150 calli in each medium, the mean growth rate values being shown in Table 1.

Protein and carbohydrate contents were measured in the culture media mentioned above. Measurements were performed in triplicate, and the mean values are shown in Table 2.

The specific activity was determined in quintuplicate (x5) by the azocasein assay and average values are shown in Table 3. Comparison of Tables 1 and 3 clearly indicates the correlation between caseinolytic activity and specific activity, and accordingly, the culture medium M1 was selected to perform subsequent experiments.

The proteolytic activity was also evaluated in quintuplicate by performing subcultures using around 100 calli for preparing homogenates (Table 4) along a period of three months, observing maintainance of the activity, of *ca* 90% of the initial activity at the end of the assay.

Under standard experimental conditions, browning of the media due to oxidation was observed as previously reported [26]. To overcome this drawback we used the half of the reported [26] usual concentration of Cu (II) in order to prevent the action of oxidases.

The effect of a variety of divalent cations - *e.g.* Ca, Mg, Mn, Zn, Co, Ni, Cu - on the proteolytic activity on calli homogenates was also examined, in final concentrations of 1 and 10 mM in the incubation medium. The concentrations were selected in agreement with the inhibition/activation compromise effect. The extent of

proteolysis was not affected by any of the cations examined.

Likewise, anions such as sulfate, phosphate, nitrate and chloride were assayed, looking for an effect as that reported in another plant proteinase [13]. However, in this case no effect on the proteolytic activity was observed, probably due to the fact that the positive activity previously reported corresponded to an aspartic proteinase, which is not the case of bromelain.

The enzymatic preparation from calli showed caseinolytic activity at different pH values, which was maximal at *ca* pH 7 and showed slight variations between pH 6 and 8, decreasing to 48 % at pH 10. When using azocasein as substrate the range of higher proteolytic activity was about pH 6, while in the case of the azocoll the pH optimum was slightly shifted to pH 5 followed by an abrupt decrease of activity toward alkaline pH values (Fig. 1). Furthermore, *ca* 40 % activity was obtained at pH 10 with azocasein and casein, but only a 10 % with azocoll at pH 9.

The behaviour of the enzymatic preparation at different temperatures is shown in Fig. 2. A maximum of activity was achieved at 60°C, and a slight activity (11 % of the maximal value) was observed at room temperature. A rapid decrease of activity was shown over 65°C.

The enzymatic preparation showed high stability at moderate temperatures, *e.g.* up to 50°C it maintained *ca* 75 % of the initial activity for 120 min. At higher temperature (70° C) the activity showed a hyperbolic decay, showing inactivation from *ca* 90 min on (Fig. 3).

Sugar content measured in the calli extract was 2.55 mg/ml. The high value of total sugar together with a protein content of 0.48 mg protein/ml of enzymatic preparation suggests the occurrence of a glycoprotein, clearly discarding the possibility of contamination. These findings are in agreement with a previous report on the glycoprotein nature of the stem bromelain obtained from the pineapple plant [14].

Specific inhibitors, such as PMSF (phenyl methyl sulphonyl fluoride), *o*-phenantroline, pepstatin (pepstatin A) and E-64 [*L-trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane] were assayed in order to identify the catalytic type of the active site of the proteases. The experimental values revealed that the enzymatic activity was 50 % inhibited by PMSF and 100 % by E-64, which is a class-specific inhibitor of cysteine proteinases (Table 5). Then, this enzyme preparation from "ananá" callus belongs to the same group of enzymes (cysteine proteinases) as those obtained from the naturally-occurring pineapple plant [13], *e.g.* pineapple bromelain. After these findings the enzyme preparation described herein was referred to as callus bromelain.

Bromelains from fresh "ananá" plant material were obtained. In fact, as in other *Ananas* species and varieties the fruit, unripe and ripe, the juice and the entire plant contain bromelains. Unlike papain, fruit bromelain does not disappear as the fruit ripens in agreement with previous reports on other *Ananas* species. The highest concentration occurs in the center of the lower portion of the mature stem.

Electrophoretic profiles were analysed for callus bromelain using the standard slab-gel techniques as described for pineapple proteinases [12]. The electrophoresis of callus bromelain resembled stem bromelain obtained from fresh pineapple in the physicochemical properties, the results being also similar to those previously reported for partially-purified crude stem bromelain (Mr, pI and glycoprotein nature) [14]. The basic stem bromelain was the major constituent, followed by ananain, comosain and traces of the acidic fruit bromelain. The Mr ranged from 23,000 to 31,000 and three distinct groups were obtained accounting for pI 4.5, pI>10 and nearly 9.5, in agreement with previous detailed reports [14]. Ananain is also the second most abundant endopeptidase of pineapple stem extract, which was first detected by its activity against Z-Phe-Arg-NHMec, which is scarcely hydrolysed by stem bromelain [12]. Stem bromelain, fruit bromelain and ananain have been shown to be immunologically distinct on the basis of previous double-immunodiffusion studies [13].

More accurate techniques were now also used, such as automatic fast system for SDS/PAGE and IEF (see Experimental) as well as capillary zone electrophoresis. Even though additional constituents were detected, they were present in both callus bromelain and plant bromelain.

Callus bromelain resembles stem bromelain obtained from fresh pineapple stem in the electrophoretic profile, physicochemical properties, pI, constituents and catalytic properties. Callus bromelain has been here assayed with protein substrates such as casein, azocasein, azocoll as described previously [12]. The specific modified protocols are indicated in the experimental section. As it is known, a sensitive and convenient leaving group for substrates of cysteine endopeptidases is 7-amino-4-methylcoumarin (-NHMec). Z-Arg-Arg-NHMec was found [14] to be an excellent and sensitive substrate for stem bromelain (EC 3.4.22.32), and to a less extent for comosain [12, 13], while fruit bromelain (EC 3.4.22.33) has a markedly different substrate specificity (Bz-Phe-Val-Arg-NHMec) compared to that of stem bromelain [13]. In terms of specificity for the hydrolysis of amide bonds, ananain (EC 3.4.22.31)and fruit bromelain (EC 3.4.22.33) are typical members of the papain family, preferring hydrophobic residues in P2 (in the recognized terminology [11]). Stem bromelain is

unusual, apparently requiring Arg in both P1 and P2 for
the efficient cleavage of small substrates, even di-Lys-containing substrates being unaffected.

Azocoll is a good substrate for many endopeptidases. The products of hydrolysis, *e.g.* the azo dye coupled to the protein, are detected spectrophotometrically after separation from the substrate by centrifugation. The assay of azocasein is also based on the formation of an azo dye by coupling of His and Tyr chains of casein with diazotized sulfanilic acid in alkali. This azo coupling provides a yellow colour on the protein. Proteolytic degradation yields trichloroacetic acid-soluble peptides that are quantified by A366. This method is very resistant to interference and was therefore here used because of being suitable for the detection of proteolytic activity in crude samples. The respective rates of casein, azocasein and azocoll hydrolyses were here examined over the pH range 4.0-10.0, which was chosen because of being the pH optima of the pineapple proteinases.

Concerning the inhibition profiles, both stem and fruit bromelains have been reported [14] to be unusual among cysteine endopeptidases of the papain family in their resistance to inhibition by chicken cystatin, glycyl endopeptidase being the only other known example. Stem bromelain is also unusual in its very slow rate of inactivation by E-64, and in this respect it appears to be unique among the papain homologs.

Cysteine proteinases comprise a family of enzymes widely distributed in nature. Several have been isolated from a number of plant sources, *e.g.* papain, actinidin, proteinase Ω, chymopapain and stem bromelain, and in some of these cases the complete amino acid sequence is known. Concerning the pineapple proteinases only the amino acid sequence of stem bromelain has been determined [27] and has shown this enzyme to be a member of the papain family. A partial *N*-terminal sequence has been reported for fruit bromelain [28] and recently the first twenty *N*-terminal residues of ananain and comosain have been determined [7]. According to these results the pineapple endopeptidases are closely related in both an evolutionary and structural sense, no unequivocal sequence differences being found between those from fruit and stem bromelains, and only two different residues out of 20 between ananain and comosain, and stem bromelain [14]. Amino acid sequence studies supported by CD analysis [16] suggest that the pineapple endopeptidases are evolutionary more closely related to each other than to other members of the papain family, including the self papain, suggesting relatively recent divergence. In fact, the spectral characteristics of stem bromelain belongs to the a + b protein class, as other cysteine proteinases do, showing however a new third spectral class of circular dichroism (CD) within the family of cysteine proteinases [16]. The high identity of the sequences as mentioned above is a clear indication that the polypeptide chains share a common folding pattern, the bromelain CD curve being however distinguishable from those reported for papain and proteinase Ω, on one hand, and that of chymopapain, on the other [16]. This is in agreement with an earlier report [27] that the active-site geometry of stem bromelain is altered with respect to that of papain.

In summary, the results obtained in this paper reveal that the pineapple calli are able to produce higher proteolytic activity than *in vivo.* Tissue culture conditions were improved through modifications of the culture media, from which the most suitable was selected according to specific activity , and protein and carbohydrate contents. Subcultures were equally evaluated prior to selection. Profiles of pH, temperature, enzyme stability, efects of anions and cations were also determined. Substrate-specificity, catalytic behaviour and physicochemical properties obtained herein for callus bromelain revealed that callus culture offer an efficient option for obtaining good rates of bromelain. The advantages of tissue culture over naturally-growing plant is the independence of technical and economic problems associated with cultivation including changes of weather and crop diseases, and heterogeneity in source material and variation in product content [22].

In this case, callus culture of “ananá” provides a continuous, aseptic and homogeneous supply of this not easily avalilable tropical plant material, and a convenient source for callus bromelain in a uniform physiological state, being also suitable system for studies on these proteinases. Furthermore, the similarity between callus bromelain and stem bromelain is of special interest. Commercial stem bromelain (EC 3.4.22.32) is used widely in industry [29], while fruit bromelain is not commercially available despite the large quantities of waste pineapple fruit portions at pineapple canneries [29].

Our enzymatic and electrophoretic data clearly show that there are distinct cysteine proteinases present in callus bromelain, similar to those detected in fresh pineapple stem bromelain (stem bromelain, ananain, comosain and traces of fruit bromelain [13]). Moreover, these findings are supported by our recent results [30] referred to the determination of these pineapple proteinases performed for the first time by Capillary Zone Electrophoresis, not only those expected peaks being observed as mentioned above but also other in small amounts, probably detected due to the extremely sensitivity of this technology. Similar CZE chromatograms were obtained for both callus bromelain and fresh stem bromelain, as will be published in detail

elsewhere. These additional peaks can be attributed to new unknown enzymes, to heterogeneities of glycoprotein components or/and to other forms of the known enzymes. Further studies are being carried out in our laboratories.

The main goal is the development of optimum production medium, satisfactory yield, reproducibility and particularly the obtention of the same catalytic type of protease as that described for plant bromelain, in particular that of industrial use.

EXPERIMENTAL

All reagents were purchased from Sigma Chemical Co. (St. Louis, Mo. USA). Pineapple stem material was obtained esentially as described previously [12].

Plant Material. Fresh material and plantlets were identified as *Ananas comosus* (L.) Merr. (Bromeliaceae) and a specimen was deposited in the Herbarium (BAFC) of the Laboratorio de Plantas Vasculares, Facultad de Ciencias Exactas y Naturales (FCEN), Universidad de Buenos Aires (UBA).

Source Material for preparing the callus culture. A market fresh *A. comosus* "ananá" ripe fruit was used as source material for the crowns from which dormant buds were isolated. The buds axillary to the leaves were excised devoid of adjacent tissue and further surface-sterilized by soaking in 70 % EtOH followed by desinfection in a commercial 12% NaOCl solution. Drops of Tween 20 were added over a period of 10 min under continuous stirring. After rinse several times in sterile distilled H2O, they were cultured on the basal medium supplemented with growth factors as indicated below.

Medium Culture. It contained saline solution and vitamins of Murashige and Skoog [31] with copper concentration reduced to the half of the original medium, sucrose (30g/l), agar (7g/l) supplemented with 10 ppm of α-naphthaleneacetic acid (ANA), and different concentrations of cytokinin. Medium 1 (M1) consisted of ANA and 0.5 ppm of benzyladenine (BA), Medium 2 (M2), ANA and 0.25 ppm BA, Medium 3 (M3), ANA without cytokinin (BA), and Medium 4 (M4), ANA and 0.5 ppm of kinetin (K).

Culture conditions. Tissue cultures were maintained at 24± 1°C in a 16h light/8 h dark photoperiod (approx. 800 lux day-light fluorescent tubes). The resultant callus cultures were routinely subcultured at 30-day intervals.

Statistical analysis of Culture growth. The results were analysed by the Student's *t*-test for growth index on the basis of the initial and final calli mass fresh weight.

Capillary Electrophoresis. Capillary Zone Electrophoresis was performed in an automated capillary electrophoresis instrument BioFocus 3000 from Bio-Rad Instruments, Hercules, California, USA, using a diode-array UV detector.

Electrophoretic profiles. Standard SDS/PAGE was carried out as previously described [12]. Gels were stained with Ag and also with Coomassie Blue.

Also the automatic electrophoresis instrument PhastSystem (Pharmacia Biotech, Uppsala, Sweden) with automated sample application and PhastGel Media for SDS gradient and isoelectric focusing (IEF) was used. A ready to use PhastGel Blue R staining tablets (Coomassie Blue R-350 stain for use with PhastGel media and PhastSystem) were used for the staining procedures. The PhastGel Gradient was Grad 10-15, which has a continuous acrylamide gradient in the range 10 to 15% for a separation range (kDa) 10-250. PhastGel IEF media of polyacrylamide gels containing 2-6% Pharmalyte as carrier ampholyte in order to generate linear pH gradients across the entire pH range and also stained with R-350 were utilized for IEF.

Extraction and determination of proteases. Callus bromelain. The acetonic powder of the calli was prepared with acetone at -20°C in a 25 % w/v rate, and further extracted with 0.1M phosphate buffer, pH 6.0, containing 5 mM disodium EDTA and 10mM 2-mercaptoethanol. The insoluble material was removed by centrifugation at 10.000 *g* at 4°C for 30 min. The supernatant was used for enzyme assays. This enzymatic preparation contained 3 g of acetonic powder per 100 ml solution.

Proteolytic activity. Kunitz's method [32] was used with casein. The incubation mixture consisted of enzyme preparation (0.5 ml), phosphate buffer (0.5 ml), 5 mM EDTA (0.1 ml) and 1 % casein solution (1 ml) in phosphate buffer containing 1 mM 2-mercaptoethanol. After 20 min the reaction was stopped by addition of 5 % trichloroacetic acid (TCA, 3 ml). The mixture was filtered, and the absorbance was read at 280 nm.

Proteolytic activity was expressed in units (U). One unit of proteolytic activity was defined as the amount of enzyme required to produce a change of 0.0001 absorbance unit per min at 280 nm under the assay conditions (20 min at 37° C).

The method of Rowan *et al.* [13] was used with standardized azocasein, consisting of incubation of the enzyme solution (130 µl), addition of the corresponding buffer (120 µl) for maintaining the respective pH, supplemented by 5 mM cysteine. The reaction was started by the addition of 2 % azocasein (250 µl) and stopped after 30 min incubation with 6% TCA (1.2 ml). Longer times of incubation may increase sensitivity. It was centrifuged and the absorbance of the supernatant was measured at 366 nm.

The method of O'Reilly *et al.* [13] was used with azocoll. Freshly prepared azocoll (5 mg) was placed in a test tube to which a suitable dilute protease solution (0.5 ml) dissolved in the buffer was added. After a 10

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min- incubation at 37 °C with manual stirring at intervals of 5 min, the reaction was stopped by the addition of H_2O (3 ml) at 4 °C. The content of the tubes was vigorously mixed and filtered through Whatman 1 filter paper. Agitation improves linearity of the assay
The absorbance of the solution was measured in a Shimadzu spectrophotometer. The protein concentration was determined by the method of Bradford [34], which was selected because EDTA and phenolics do not interfere with the reaction.
Proteolytic activity as a function of the pH and temperature. Casein solutions were used in the forementioned conditions in order to study the proteolytic activity after changing pH and temperature. These casein solutions were prepared in (a) citric acid/citrate buffer, pH 5.5; (b) phosphate buffer, pH 5.8 to 7; (c) Tris buffer, pH 7 to 9; (d) carbonate/bicarbonate buffer, pH 9 to 10; (e) 0.1 M each buffer mentioned above, containing 5 mM EDTA.

The enzymatic behaviour was determined at pH 7.0 and temperatures varying from 25°C to 70°C.
Thermal stability of the enzymatic solution. The enzymatic preparations were incubated for 5 to 120 min at a temperature range from 40 to 70°C. At the end of each time period, samples were maintained in an ice bath until assayed for caseinolytic activity.
Inhibition assays. The enzymatic preparation (0.5 ml) was incubated with a variety of inhibitors (10 µl each) for 30 min at 30° C, such as pepstatin A, E-64, PMSF and *o*-phenantroline. A control devoid of inhibitor was treated in the same way. Subsequently,
samples were worked-up as indicated for standard assays.
Sugar content. Total sugars were determined by the method of Dubois *et al.* [36].
Abbreviations: Pepstatin: pepstatin A, isovaleryl-Val-Val-AHMHA-Ala-AHMHA [AHMHA = (3S,4S)-4-amino-3-hidroxy-6-methyl-heptanoic acid]; E-64: *L-trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane; PMSF: phenylmethylsulphonyl fluoride [35].

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Table 1: Proteolytic activity and growth rate in different culture media

Media	Specific activity (Uazoc./mg)
M1	5.38
M2	5.06
M3	4.68
M4	4.52

Growth rates refer to mean values

Table 2: Protein and Carbohydrate contents in different culture media

Media	Proteins (mg/ml)	Carbohydrates (mg/ml)
M1	0.485	2.55
M2	0.280	2.32
M3	0.180	2.49
M4	0.420	3.00

Protein and Carbohydrate contents refer to mean values (see Experimental).

Table 3: Specific activity determination in the different culture media of calli tissue

Inhibitor	Concentration	Relative activity (%)
None	**-**	**100**
PMSF	**1.0 mM**	**51**
Pepstatin A	**10.0 µM**	**100**
***o*-phenantroline**	**10.0 mM**	**96**
E-64	**10.0 µM**	**1.2**

Specific activity referes to mean values.

Table 4: Proteolytic activity in different subcultures

Culture number	Specific activity (Uazoc./mg)	Time (months) *
1	5.40	1
2	5.15	2
3	4.80	3

* The subculture was realized every 30 days.

Table 5. The effect of the different inhibitors on the proteolytic activity

Media	Activity	Growth rate
M1	++++	1.77 ± 0.42
M2	+++	1.47 ± 0.34
M3	+++	1.15 ± 0.37
M4	++	1.05 ± 0.28

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Fig. 1: pH Profile of the callus proteolytic activity

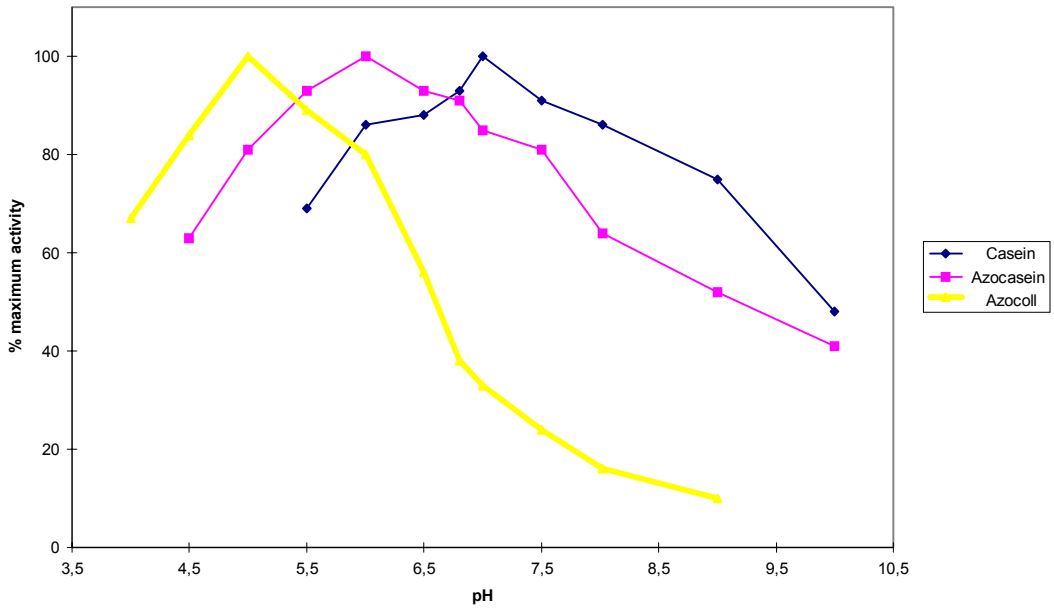

Fig. 2 Caseinolytic activity as a function of temperature

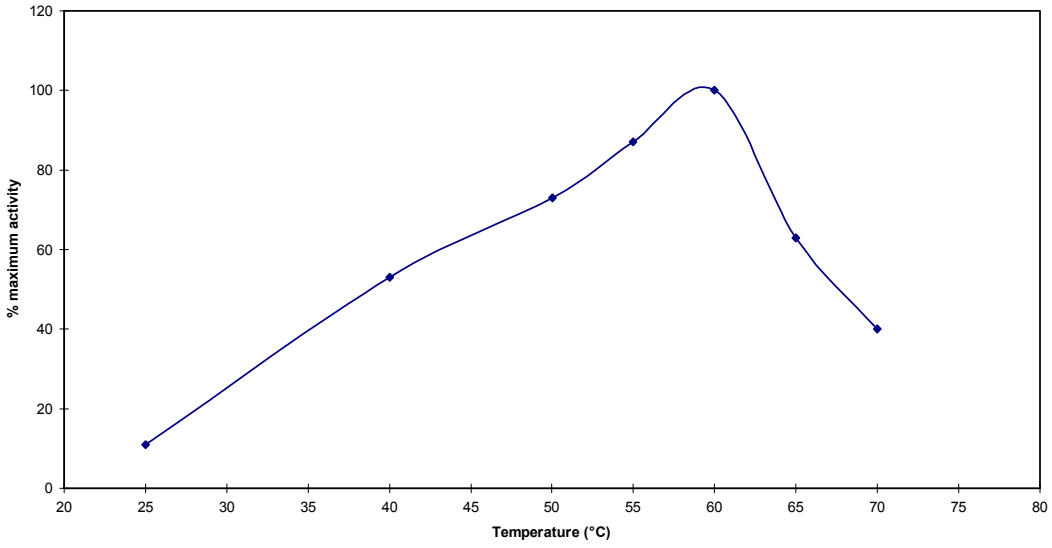

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Fig.3: Callus enzyme inactivation by temperature