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Enzymes of *Opuntia ficus-indica* (L.) Miller with Potential Industrial Applications-I

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Abstract

We report on the screening of different enzymes such as arylamidases, lipases, proteinases, and glucosidases in plant extracts of the Cactaceae family, genus *Opuntia,* as well as on a newly purified plant proteases from *O. ficus-indica* fruit extracts. These proteinases showed the maximum activity at pH 5.2 and 55°C and FTC-casein was the best of the screened substrates. Proteolytic activities were activated by anti-oxidant compounds and by some divalent cations. These proteinases were efficiently inhibited by cystein proteinase inhibitors and by 1,10-phenanthroline. The estimated M_r for the main proteolytic activity was about 23.2 kDa. The results on milk clotting characteristics suggest a potential use of the fruit cystein enzymes of this plant in dairy industries.

Index Entries: Proteinases; proteinase inhibitor; arylamidases; lipases; glucosidases; collagenase; FITC; FTC-protein; milk clotting.

Introduction

Plants are important sources for different industrial enzymes, but for several reasons (e.g., seasonability) the number of enzymes from plant origin used by industry is rather low. Extracts of some plants containing several kinds of enzymes may be used as crude extracts or as a source of enzymes of high value products for industrial processes. The need for new specific enzymes, and considering that only a small percentage of plant species have been tested, implies that plant extracts must be screened for their enzyme production and activities.

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Proteinases are the most important plant enzymes used in the food, pharmaceutical, detergent, leather, and wool industries *(1)*. The most widely used plant proteinases are papain from *Carica papaya*, bromelain, ananain and pinguinain from *Ananas comosus* and *Bromelia pinguin*, ficin from *Ficus sp*. *(2)*, cinarin (or ciprosin) from *Cynara cardunculus (3,4)*, and actinidin from *Actinidia sp*. *(5)*.

Opuntia is a genus of the Cactaceae family that grows worldwide. The most usual and studied species of this genus is *Opuntia ficus-indica* (L.) Miller, probably native from Mexico *(6)*. Owing to its strong adaptability it grows in different regions of the world and can be found in North, Central, and South America; the Mediterranean countries; North and Central Africa; the Middle East; and Australia *(7)*. In countries such as Italy, Spain, Mexico, Chile, Brazil, and Argentina, *O. ficus-indica* is cultivated for commercial purposes, particularly as food and forage *(8)*. The fresh fruit have been utilized as a beverage *(9)*. Fresh, dried, or cooked fruits have also been used as animal and/or human food *(10,11)*. The levels of minerals, vitamins, water, polysaccharides and fibers are higher than usually observed in most plants *(8,10,11)*. The agro-industrial potentiality of *Opuntia ficus-indica* is large: canned juice *(8)*, jam, jelly, molasses-type candies *(12)*, wine *(13)*, vinegar *(12)*, and cooking oils *(8)*. There are several references on medicinal uses of this plant, mainly against cold, wounds, ulcer, allergic reactions, syphilis, whooping cough *(14)*, prostate hypertrophy *(15)*, hyperlipidemiae, and obesity *(16)*. Some authors suggest its use in mixed formulations with other plant extracts: against anti-retroviral activity; virus diseases like herpes simplex, influenza, and HIV; antiautogenous diseases; carcinostatics; anti-oncogenics; preventing flacherie of silkworm; treating the wrinkling of human skin and sun overexposure; controlling some soft-bodied insects; and lysis of bacteriophages.

Literature is scarce on enzymes from *Opuntia sp*. except for invertase *(17)*, glucosidases *(18)*, and enzymes from CAM metabolism *(19)*. The aim of this work was the screening for new and potentially important enzymes for industry, in plants of the genus *Opuntia*, especially *O. ficus-indica*, and a partial characterization of its main proteolytic activity.

Materials and Methods

Chemicals

Casein and fluorescein isothiocyanate (FITC) and hide powder azure (HPA) were from Fluka (Switzerland); hemoglobin, casein, albumin, gelatine, collagen, collagenase, and *N*(3'-(2-furylacryloil))-leucylglycil-prolylalanina (FALGPA) from Sigma (Spain); molecular protein weight markers from Pharmacia (Sweden); and the API Zym kit was from Bio Mérieux (France). Skimmed milk (powder) is Molico from Nestle. The milk from sheep, cow, and goat were kindly supplied by Queijo Saloio (Torres Vedras, Portugal). UHT milk is from Lactogal (Portugal). All other chemicals used were of analytic grade or the best available commercial products.

Biological Materials

Opuntia species were collected in JMAT garden (Lisbon, Portugal) and *O. ficus-indica* was also collected in Cotovia (Sesimbra, Portugal) from August to October, and cooled on dry ice. The cladodes and the fruits were stored at –70°C, until use. The studied species were *O. ficus-indica*, *O. paraguaiensis*, *O. nonacantha*, and *O. maxima (20)*. The biological materials were stored at the Herbarium of the Agrarian Tropical Garden Museum (LISJC, Lisbon).

Methods

All assays were performed in duplicate and in sets of three samples.

Preparation of Enzymatic Extracts

Frozen cladodes and fruits of *Opuntia sp*. were homogenized in cold 50 m*M* Tris/HCl buffer pH 8.0. Homogenates were incubated at 4°C for 30 min with mild agitation. The extracts were collected by centrifugation at 12,000*g* for 20 min at 4°C.

API ZYM Test

The Kit Api Zym was used for semiquantitative (nmole range) detection of some lipase, arylamidase, proteinase, glucolytic, alkaline, and acid phosphatase type enzymes *(21)*.

Proteinase Assays

1. The hydrolytic activities of different proteins conjugated to FITC were assayed by the fluorimetric method of Twining *(22)* slightly modified *(4,23)*. The FTC-protein substrates had 0.8 mg of protein/mL. One unit of proteolytic activity was defined as the enzymatic activity that increases by one unit the emitted fluorescence at 525 nm, after 30 min of hydrolysis at 37°C under the specified assay conditions (*23*). This is equivalent to a release of 1 ng of protein/min from the substrate FTC-protein.
2. Method of Lamb *(24)* was used to estimate the effect of the extracts on hide powder azure (HPA) and for the evaluation of the extracts as bates *(24,25)*. This method was performed in the range 0.2–1.0 mg/mL of collagenase type enzymes.
3. Method of Van Wart and Steinbrink *(26)* with the short synthetic peptide FALGPA as substrate was used to estimate collagenase activity in extracts.

Protein Determination

The method of Bradford *(27)* was used for protein determination in the range 0–10 μg of protein with bovine serum albumin (BSA) as standard.

Selection of Optimal FTC-Protein Substrate

FITC was conjugated with casein, albumin, collagen, and denatured hemoglobin yielding the following substrates FTC-casein, FTC-albumin, FTC-collagen, and FTC-hemoglobin. The incubation buffers were used according to the stability and optimum pH and temperature of each proteolytic activity *(22)*, and the assay was done as described above *(4,23)*. The FTC-protein allowing the highest activity was considered as the optimum substrate and its enzymatic activity value was taken as 100%.

Optimum pH and Stability to the pH of the FTC-Caseinolytic Activities

The 0.2 *M* incubation buffers used were sodium acetate (pH 3.6–4.4), sodium citrate (pH 4.4–6.0), sodium phosphate (pH 6.0–7.4), Tris/HCl (7.4–8.5), and sodium borate (8.5–9.0). To determine the stability to the pH, extracts were maintained in 50 m*M* of each buffer solution at 20°C for 24 h and at 4°C for 7 d. The remaining activities were measured after appropriate dilution in 0.2 *M* citrate buffer pH 5.2 *(4,22,23)*.

Optimum Temperature and Stability to the Temperature of the FTC-Caseinolytic Activities

Samples were preincubated for 30 min in 0.2 *M* citrate buffer, pH 5.2, at 4, 8, 20, 25, 30, 37, 45, 50, 55, 60, and 65°C. The residual proteolytic activity was then assayed by the selected method *(23)*.

Stability to the temperature was tested in 50 m*M* Tris/HCl buffer, pH 8.0, and in 50 m*M* sodium citrate, pH 5.2, at –70, –20, 4, 20, 25, 30, 37, and 45°C for 24 h.

The optimum temperature for long-term storage of the proteolytic samples was also studied from –70 to 4°C.

Effect of Some Cations and Anti-Oxidant Compounds on FTC-Caseinolytic Activities

Magnesium (Mg), calcium (Ca), copper (Cu), cobalt (Co), manganese (Mn), and zinc (Zn) and the thiol reducers β-mercaptoethanol (β-ME), reduced glutathione (G-SH), dithiothreitol (DTT), and cystein (Cys) were tested. In the proteolytic assay, their final concentration was 2 m*M* in either 0.2 *M* Na citrate or 0.2 *M* MES buffer, pH 5.2.

Effect of Proteinase Inhibitors on FTC-Caseinolytic Activities

The final concentration of each inhibitor in the preincubation mixtures was in the range recommended in ref. *28*. The inhibitors used were PMSF, leupeptin, aprotinin, E-64, cystatin, iodoacetamide, pepstatin-A, EDTA, EGTA, and 1,10-phenanthroline. The active fruit extracts of *O. ficus-indica* were incubated for 15, 30, 45, 60, 90, and 120 min in the presence of each inhibitor. Residual proteolytic activities were calculated after performing enzymatic assays, as described above *(23)*.

"In Situ" *Detection of Proteolytic Activities on PAGE*

The proteolytic activities were detected "*in situ*" by zymogram analysis on 10% SDS-PAGE containing 0.05% (w/v) casein *(29)* or gelatin *(30)*. Before application, samples were diluted in the SDS sample buffer *(31)*, but not boiled. Electrophoresis was run in a vertical mini-gel system Hoeffer SE-200 (Pharmacia) and performed as usual *(31)*, at 70 V for 4 h. Gels were processed as mentioned by standard methods used *(29,30)*, but the activating buffer was 0.2 *M* sodium citrate pH 5.2, 10 m*M* β-mercaptoethanol (β-ME), and 2 m*M* $CaCl_2$. The gels were stained (with Coomassie Blue R-250) according to the standard procedures *(29,30,31)*.

Milk Clotting Activity

One half milliliter of *Opuntia ficus-indica* fruit extracts (0.38 mg/mL) was added to 4.5 mL of each type of milk. The assayed milks were skimmed milk (Molico) containing 10 m*M* $CaCl_2$, bovine pasteurized milk, and milk from cow, goat, and sheep origin. The assays were performed at 32°C according to the modified method of Berridge, IDF110-A Norma *(32)*. The clotting time was the time (in min) required to clot milk.

Gel Filtration

Extract of fruits of *O. ficus-indica* was dialyzed against 50 m*M* sodium phosphate and 150 m*M* sodium chloride buffer pH 7.0 (gel filtration buffer) and concentrated 5X by ultrafiltration on an Amicon System (PM10 membrane), at 4°C; 0.1 mL of the sample was applied to the Superdex 200HR column (Pharmacia) previously equilibrated with gel filtration buffer *(33)*. The run was performed in the same buffer, at 0.3 mL/min, 4°C, and fractions of 0.25 mL were collected and assayed for FTC-caseinolytic activity *(23)*. Calibration curve was made after the separation run of some low- and high-molecular-weight protein markers (Pharmacia) as above *(33)* and the molecular weight (as M_r) of the proteinases was estimated.

Results and Discussion

Screening of Several Enzymatic Activities

The API Zym kit was tested in extracts of cladodes and fruits of the species of *Opuntia* studied. No significant enzymatic activity differences were observed among *O. maxima*, *O. ficus-indica*, and *O. paraguaiensis*. Fruit extract from *O. nonacantha* is difficult to obtain, as this fruit is very dry and fibrous. On the other hand, almost all enzymatic activities were higher in unripe fruits than in ripe fruit and ovaries. As *O. ficus-indica* is the most abundant and widely dispersed species of the genus, it was chosen for the subsequent assays. The results obtained with the API Zym test for *O. ficus-indica* fruit extracts are shown in Table 1.

Table 1
Screening of Enzymatic Activities on 50 µL (15 µg of protein) of Fruit Extract of *Opuntia ficus-indica* with API Zym Kit (BioMérieux)

Enzymatic activity detected	pH and color of the positive reaction	Enzyme, nmoles
Alkaline phosphatase	8.5, violet (+++)	≥ 40
Esterase lipase (C4)	6.5, violet (+)	5
Leucine arylamidase	7.5, orange (+)	10
Valine arylamidase	7.5, orange (+)	10
Trypsin	8.5, orange (+)	10
Acid phosphatase	5.4, violet (+)	5
Naphthol-AS-BI-phosphohydrolase	5.4. blue (++)	30
β-Galactosidase	5.4, biolet (+)	5
N-acetyl-β-glucosaminidase	5.4, brown (+)	10

Assay of Proteinases

The ratio proteolytic activity/wet weight of plant material was eight times higher in fruits (approx 6500 U/mg of fruit) than in cladode extracts. The proteolytic specific activity is also 12 times higher in fruit than in cladode extracts, at the level of 20300 U/mg protein. The cladode extracts are much more viscous and more difficult to work with.

Optimum FTC-Protein Substrate

Figure 1 shows that FTC-casein is the best of the FTC-protein substrates tested on proteinases of extracts from unripe fruits of *O. ficus-indica*, under the assay conditions *(23)*. The proteolytic activity on FTC-collagen was less than 4% and the control reaction had a high background.

Other Types of Proteinases

With FTC-casein as substrate, and based on the proteolytic assay method *(22,23)*, other types of proteolytic activities were screened on fruit extracts of *O. ficus-indica*: 6.8% of elastase activity (pH 8.8) as well as 4.6% of termolysin, chymotrypsin/trypsin (pH 7.8), and 3.6% of protease from the subtilysin (pH 7.6) were found. Hide powder azure (HPA), the denatured collagen in the form of "hide powder" linked to the dye Remazol Brilliant Blue, have also been considered good substrates for many endopeptidases, e.g., collagenase type enzyme *(24,35)*. Collagenolytic activities were not detected by the assay methods used *(24,26)* on *O. ficus-indica* tested extracts. The value obtained when the FTC-collagen was used as substrate (Fig. 1) could be due to the extreme sensitivity of the fluorimetric assay, which enables the detection of a few nanograms of different kinds of endopeptidases *(22)*.

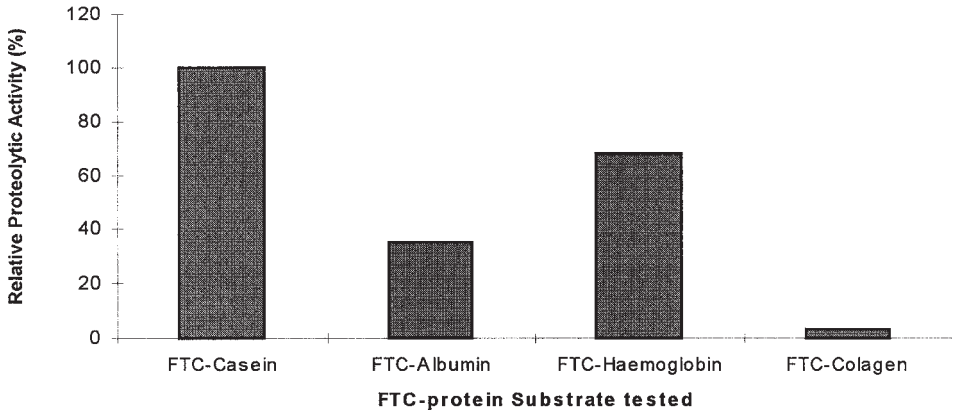

Fig. 1. Screening for the optimum FTC-protein substrate by the modified method of Twining *(23)*. The highest proteolytic activity was obtained with FTC-casein and its value has been taken as 100%.

Effects of pH on Proteolytic Activity and Stability

FTC-caseinolytic activities of the *Opuntia ficus-indica* fruit extracts were detected over a broad pH range (4.5–7.5) and the optimum pH is 5.2. The same optimum pH value was obtained for the crude and partially purified enzyme (Fig. 2). This value of optimum pH is similar to those obtained for some aspartic proteinases *(4,23,36)*. While using different methods of assays, some cystein proteinases such as chymopapain *(37)* and actinidin *(5)* gave optimum pHs at about 6.0. The stability to the pH, at 4 and 20°C, of proteolytic activities of extract from fruits of *O. ficus-indica* are represented in Fig. 3 (24 h at 20°C) and in Fig. 4 (7 d at 4°C).

After 24 h at 20°C, the proteolytic enzymes are more stable over the pH range 4.8–6.8 (Fig. 3) and at 4°C the residual values displayed were higher than 60% over a broad pH range (pH 4.8–8.0) (Fig. 4). In order to preserve the proteolytic activities and prevent self-proteolysis, these extracts of *Opuntia ficus-indica* were stored at pH 8.0, far from the optimum pH but within the range of stability to the pH of these proteinases.

Effects of Temperature on the Proteolytic Activity and Stability

The optimum temperature for the FTC-caseinolytic activity of *O. ficus-indica* fruit extract was 55°C, as is shown in Fig. 5. The proteolytic activity values obtained at 60°C and at 70°C were less than 50% and 5%, respectively, than that obtained at 55°C. Most of the proteinases from plant origin have high values of optimum temperature and have a broad stability to the temperature, even at high temperature *(28)*.

It is evident from Fig. 6 that the best long-term storage temperature is –70°C and that at –20°C it is possible to store the extracts for 15 d with a loss of proteolytic activity lower than 10%.

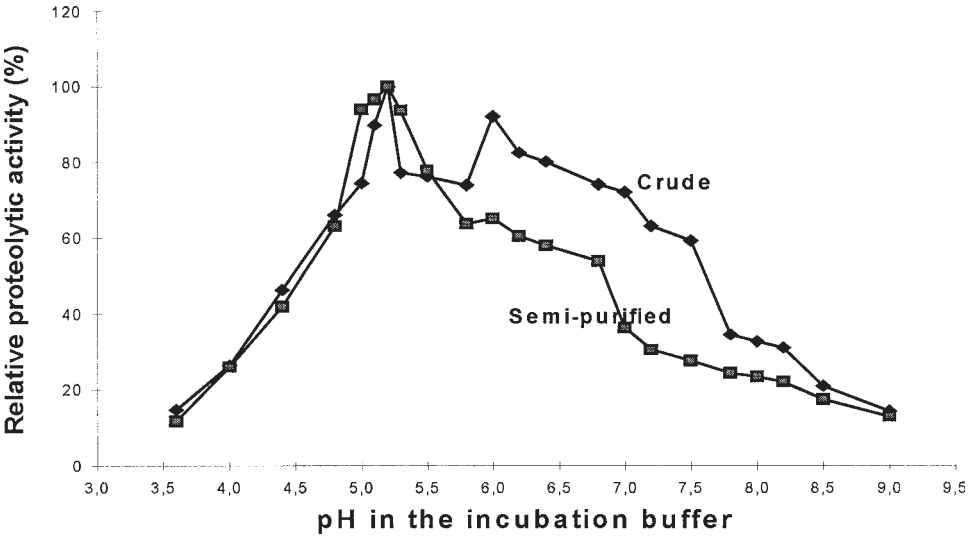

Fig. 2. Determination of the optimum pH for crude and partially purified (by gel filtration) proteolytic extracts from *Opuntia ficus-indica* fruits. The assay method used was Twining, modified (*23*). Samples were incubated with FTC-casein in 0.2 *M* buffers from pH 3.5 to 9.0 (sodium acetate for pH 3.5–4.8, sodium citrate for pH 4.5–6.2, sodium phosphate for pH 6.0–7.4, Tris/HCl for pH 7.2–8.5, and sodium borate for pH 8.5–9.0) for 30 min at 37°C, as mentioned in Methods.

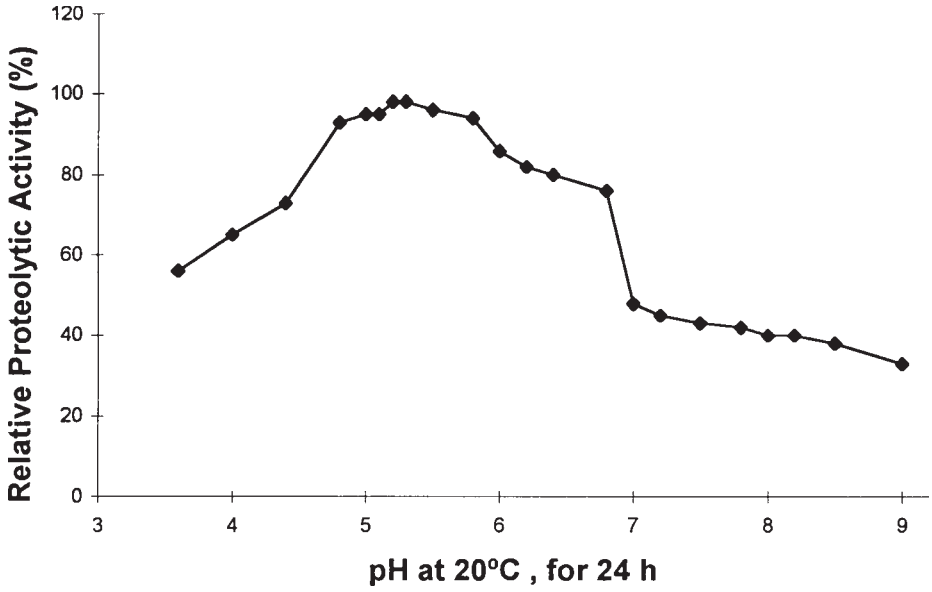

Fig. 3. Stability to the pH of the FTC-caseinolytic activities of extract from fruits of *Opuntia ficus-indica* at 20°C. Samples were incubated at 20°C (environmental temperature) for 24 h in the following 0.2 *M* buffers: sodium acetate (pH 3.5–4.8), sodium citrate (pH 4.5–6.2), sodium phosphate (pH 6.0–7.4), Tris/HCl (pH 7.2–8.5), and sodium borate (pH 8.5–9.0).

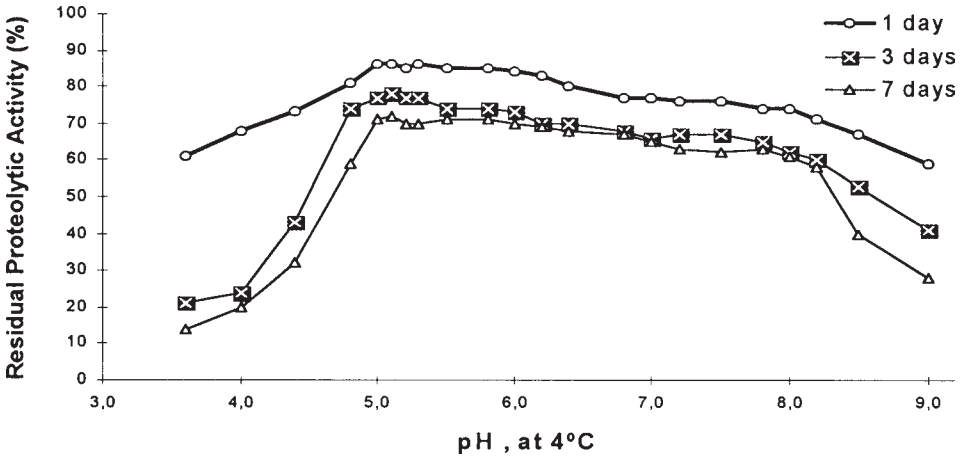

Fig. 4. Stability to the pH of the proteolytic activity of the *O. ficus-indica* fruit extracts in 0.2 *M* buffers at 4°C for 7 d. The assay method *(23)* is the same as above. The FTC-caseinolytic activity value obtained for each pH after the extraction procedure (at d 0) was taken as 100%.

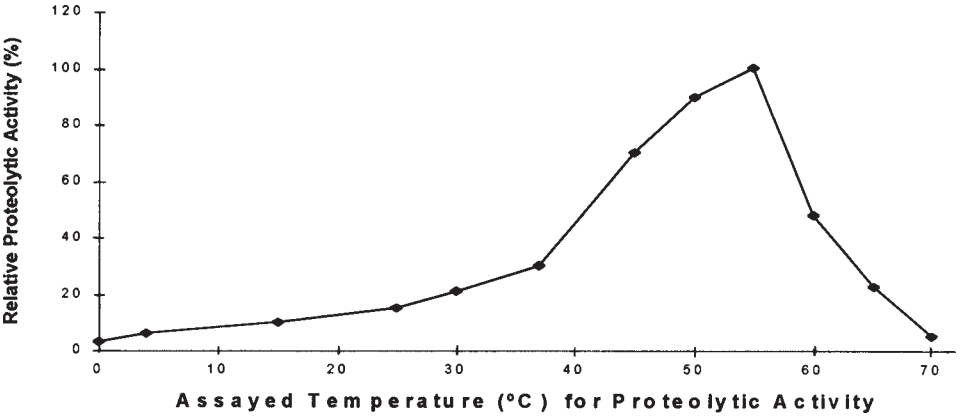

Fig. 5. Determination of the optimum temperature for the assay of proteolytic activities from *O. ficus-indica* fruit extracts *(23)*. The reaction mixtures were incubated at temperatures from 0 to 70°C in 0.2 *M* Na citrate pH 5.2 buffer for 30 min. The highest value of proteolytic activity obtained was taken as 100%.

Effects of Cations and Anti-Oxidant Compounds

Figure 7 shows the effect of some modulators (cations and anti-oxidant compounds) on these proteolytic activities. The FTC-caseinolytic activities from *O. ficus-indica* fruit extracts were strongly enhanced in the presence of the thiol antioxidant such as β-mercaptoethanol (β-ME), dithiothreithol (DTT), and cystein (Cys).

The activating effect of these thiol compounds on proteases was reported for many cystein-proteases *(5,28,29,38,39)*. The proteolytic activi-

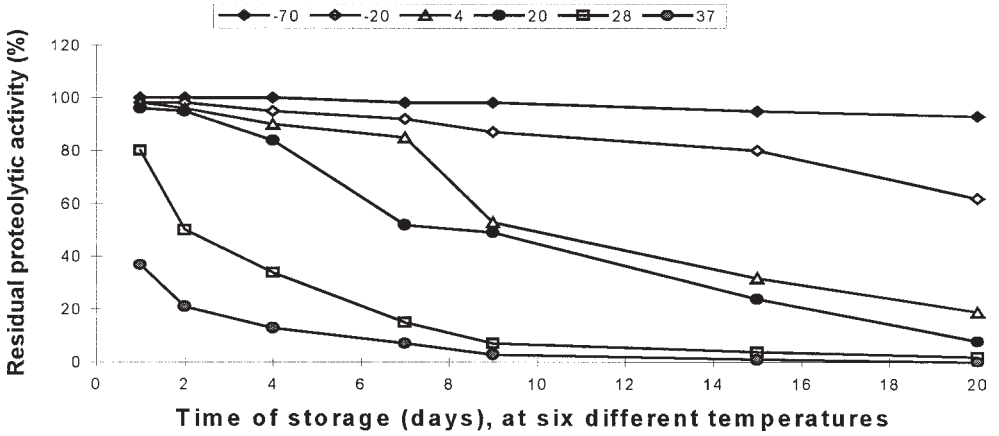

Fig. 6. Influence of the temperature of storage on the stability of FTC-caseinolytic activities of *O. ficus-indica* fruit extracts (in 50 m*M* Tris/HCl pH 8.0) during 20 d. The assayed temperatures were: –70, –20, 4, 20, 28, and 37°C, and the highest value obtained after 1 h of storage was considered as 100%.

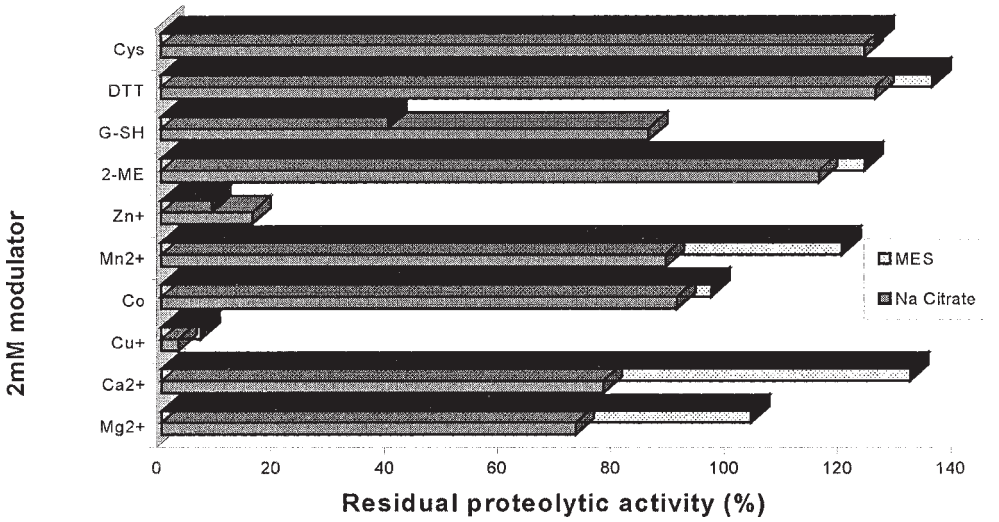

Fig. 7. Effects of some cations and thiol antioxidant on proteolytic activities of fruit extracts from *O. ficus-indica*. Modulators were incubated in the assay at a 2 m*M* final concentration both in 0.2 *M* MES and sodium citrate pH 5.2 incubation buffer. The tested modulators were magnesium (Mg^{2+}), calcium (Ca^{2+}), manganese (Mn^{2+}), copper (Cu^{+}), cobalt (Co^{+}), zinc (Zn^{2+}), β-mercaptoethanol (2-ME or β-ME), dithiothreitol (DTT), reduced glutathion (G-SH), and cystein (Cys).

ties of the extracts from fruits of *O. ficus-indica* increase also in presence of the Mg^{2+}, Ca^{2+}, and Mn^{2+} in the 0.2 *M* MES buffer at pH 5.2 but are strongly inhibited in presence of zinc or cobalt.

Table 2
Effects of Some Proteinases Inhibitors on the Proteolytic Activities of Fruit Extract from *Opuntia ficus-indica*[a]

Mechanistic class of inhibitor	Inhibitor (upper final concentration tested)	Inhibition (%)
Serine	Aprotinin, 3.8 U/μL	67
	PMSF, 1.0 m*M*	7
	Leupeptin, 100 μ*M*	70
Cysteine	E-64, 10 μ*M*	77
	Cystatin, 100 μ*M*	47
	Iodoacetamide, 100 μ*M*	71
Aspartic	Pepstatin-A, 1.0 μ*M*	48
Metalo or metalo-activated	1,10-Phenantroline, 10 m*M*	82
	EDTA, 10 m*M*	5
	EGTA, 10 m*M*	6

[a]Each inhibitor was incubated with the sample in the range of effective inhibition final concentration, for 30 min at 30°C before the addition of FTC-casein. The proteolytic assay was done as usual *(23)*.

Effect of Proteinase Inhibitors on Proteolytic Activities

Specific protease inhibitors may be used to identify catalytic groups within the active center of the proteinases *(23)*. Influence of some inhibitors on the proteolytic activities of *O. ficus-indica* fruit extract was analyzed and reported in Table 2. Only PMSF did not strongly inhibit these proteolytic activities. Some inhibitors as pepstatin-A and cystatin induced inhibitions lower than 50%. Leupeptin allowed a 30% of residual activity, but this molecule inhibits serine and some cystein proteinases. Inhibition by E-64 was obtained earlier after 10 min of incubation. Stem bromelain is the only papain homolog proteinase unusual in its very slow rate of inactivation by E-64 *(40)*. 1,10-Phenanthroline is the compound that promoted the strongest inhibition, but it is a large molecule and can obstruct the catalytic center *(36,40)*.

When extracts were incubated in the presence of EDTA and EGTA, at their effective concentration for inhibition, the residual proteolytic activities were close to 95% but metalo or metalo-activated proteinases are not activated by thiol compounds and are inhibited by EDTA or EGTA *(40)*. Probably, in crude extracts from fruits of *Opuntia ficus-indica* there are more than one proteinase or the proteinase present is of the cystein type and needs a metal ion for its effective activity.

Gel Filtration

Calibration curve was made with low-molecular-weight marker protein and the r was 0.997 (data not shown). The estimated molecular weight, in terms of M_r is about 23200 Dalton. This value is close to that reported for most cystein-type proteinases *(2,5,37–39,41)*.

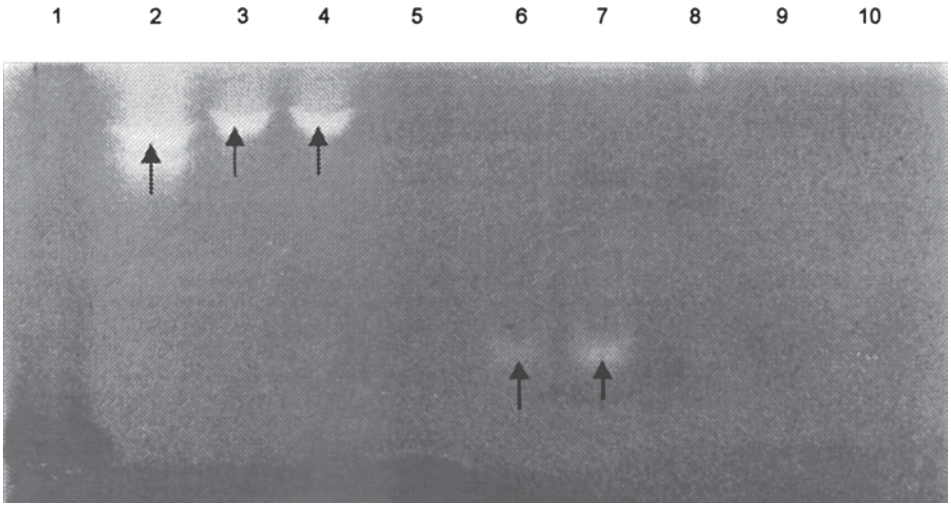

Fig. 8. 10% SDS-PAGE with 0.05% casein performed as described above *(29)*. Samples in the gel were: 1, ribonuclease and phosphorylase; 2–4, Fruit extract from *O. ficus-indica*; 5, similar to 3 and 4, but boiled at 100°C for 5 min; 6 and 7, *O. ficus-indica* fruit proteinase partially purified by gel filtration; 8, bovine serum albumin; 9 and 10, sample similar to 6 and 7, but boiled 100°C, for 5 min. The clear zones in the gel indicated by arrows, were due to the hydrolysis of the casein by the tested proteolytic samples.

In Situ *Detection of Proteinase on Casein-SDS-PAGE*

In Fig. 8, two areas of intense proteolysis (clear areas within dark background) were detected on the casein-acrylamide gel, one at about M_r 69500 Dalton and the other at M_r 29800 Dalton. The partially purified proteinases from the *Opuntia ficus-indica* fruit extract by gel filtration show hydrolysis of casein only at about M_r 29800. The higher-molecular-weight proteolytic activity present in the fruit extract could be due to some aggregation phenomena.

Potential Industrial Applications

Dairy Industry

Dairy industries have been looking for different rennets, and plants have been screened for this task. Cheeses obtained from papain, ficin, bromelain, and some other plant proteases had been referred as clot products without well-accepted organoleptic characteristics. Different types of milk were tested for clotting activity *(32)* with extracts from fruits of *Opuntia ficus-indica*. The results obtained were analyzed in terms of clotting time.

The sample used for these assays had 0.38 mg/mL of protein. The clotting times obtained were 20 min for UHT cow milk, 21 min for sheep milk, 24 min for skim milk Molico, 30 min for cow milk, and more than 45 min for goat milk.

Cheeses of cow milk prepared with ciprosin tend to taste bitter and present texture defects *(36)*. Extract from fruits of *Opuntia ficus-indica* seems to be a good source of milk clot enzymes to be used in dairy industries as it has good smell and good texture, and the clotting times were not so far from that obtained for other plants rennets *(23,36)*. The milk clotting activities of *O. ficus-indica* fruit extracts are probably due to its proteolytic activities as it occurs with other proteinase *(3,23,36)*.

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