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# Partial Purification and Characterization of Two Proteases from *Agave fourcroydes*

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## ABSTRACT

Large amounts of wastes are produced by the henequen industry from which it may be possible to obtain products of commercial importance. Our laboratory has detected proteolytic activity in *Agave fourcroydes* (henequen) juice. The proteolytic activity is a result of two proteases of 14,500 and 12,000 daltons, respectively. Both enzymes are very stable at −20°C, when freeze-dried and in the presence of EDTA and cysteine. They differ in pH optimum and thermal stability.

**Index Entries:** *Agave fourcroydes;* proteases; protein purification.

## INTRODUCTION

Large amounts of wastes are produced by the henequen industry. The isolation of 1 kg of fiber produces 24 kg of wastes, 17 kg of which are juice. Mexico produces around 60,000 t of fiber, corresponding to one million t of agave juice. In *Agave fourcroydes* this amount of juice contains approximately 1700 t of proteins.

Proteases have received a lot of attention since they can be used in many industries. Proteases have been previously detected in agave juice

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(*1–5*) and some reports have shown that these wastes may be a useful raw material for obtaining proteolytic enzymes (*3*). However, these agave proteases have not been adequately characterized.

In Mexico, around 80% of these wastes are generated in the state of Yucatan. The potential application of these enzymes, and the lack of knowledge about them, have led us to be interested in the extraction and characterization of the proteases from the *Agave fourcroydes* juice.

## MATERIALS AND METHODS

### Biological Material

Agave juice was obtained by pressing the leaves in a roller press. The juice was collected in the presence of sufficient Tris-HCl to yield a 50 m*M* final concentration, with or without 2 m*M* EDTA and 10 m*M* cysteine. The pH was finally adjusted to pH 8.0. The juice was centrifuged at 14,000×g for 30 min and the supernatant used for activity measurements and purification procedures.

### Assay of Proteases

Proteolytic activity was measured using 2% (w/v) casein solution in 100 m*M* Tris-HCl pH 8 as substrate. The reaction mixture contained 200 μL enzyme extract and 800 μL 100 m*M* Tris-HCl pH 8 with 2 m*M* EDTA and 10 m*M* cysteine. The sample was incubated at 37°C for 2 min prior to initiating the reaction by the addition of 2 mL of casein. With activity measurements the system was always checked for linearity with time and enzyme concentration. Enzyme concentration studies were carried out with a 10 min time course. A constant volume of 200 μL of crude extracts was used for different times courses. The reaction was stopped with 4 mL of 5% (w/v) trichloroacetic acid and the mixture centrifuged at 3000 rpm for 20 min. The supernatant was assayed with Folin-Ciocalteau reagent. Blanks were used in each determination. For the time course reaction a complete mixture was stopped before the reaction began. For enzyme concentration studies a time zero blank was used for each concentration. One protease unit is defined as the amount of enzyme that hydrolyses casein to produce 1 μmol of tyrosine/min at pH 8.0 at 37°C. Specific activity is defined throughout the text as protease units, both g/fresh w or mg protein.

### Purification Procedure

Precipitation by organic solvents is a conventional method for the extraction and purification of industrial enzymes. This method is relatively cheap if a high efficiency solvent recovery is guaranteed. For this reason, we used ethanol as the first step in purification, mixing 100 mL of crude extract with 50 mL of ethanol. After ethanol addition the mixture was

stirred at 4°C for 30 min before centrifuging at 14000×g. The supernatant was discarded and the precipitate resuspended in the smallest possible volume of extraction buffer, centrifuged at 14,000×g, and applied to a G-200 Sephadex column (1.6×90 cm). The collected fractions (3.7 mL) were assayed for enzyme activity. The samples from fraction 90 to 100, and from 101 to 111, were pooled and frozen at −20°C to be used in the characterization experiments.

### Heat Stability

Thermal stability of the purified samples was determined by keeping 200 μL of the enzyme solution and 800 μL of the assay buffer at different temperatures for 30 min. Subsequently, the samples were assayed for proteolytic activity, as described previously.

### pH Optimum

Three hundred μL of the purified material were mixed with 700 μL of the assay buffer at different pHs and then treated as described above.

### Molecular Weight Determination

Andrews' method (*6*) was used.

### Protein Determination

Peterson's modification (*7*) to the Lowry et al. method was used using bovine serum albumine as standard.

## RESULTS

Proteolytic activity was assayed in the agave juice to determine the optimal conditions for the evaluation of both enzyme concentration (Fig. 1A), and time (Fig. 1B). In both cases, only the linear part of the curve was used for the rate determination.

Different specific activities were obtained with freshly extracted agave juice prepared with or without activators. Activity was 60% lower in the absence of EDTA and cysteine with freshly isolated juice. After 24 h storage at −20°C, the specific activity of the sample with activators was three fold higher (Fig. 2A), and ten days later this had increased to six fold (Fig. 2B). In general, the samples were more stable in the presence of EDTA and cysteine (Fig. 3).

Precipitation with ethanol served to concentrate the juice (Table 1). The use of a gel filtration column increased the specific activity and showed that the proteolytic activity of agave juice is caused by two proteases at least. The enzymes differ in pH optimum, thermal stability, and molecular weight.

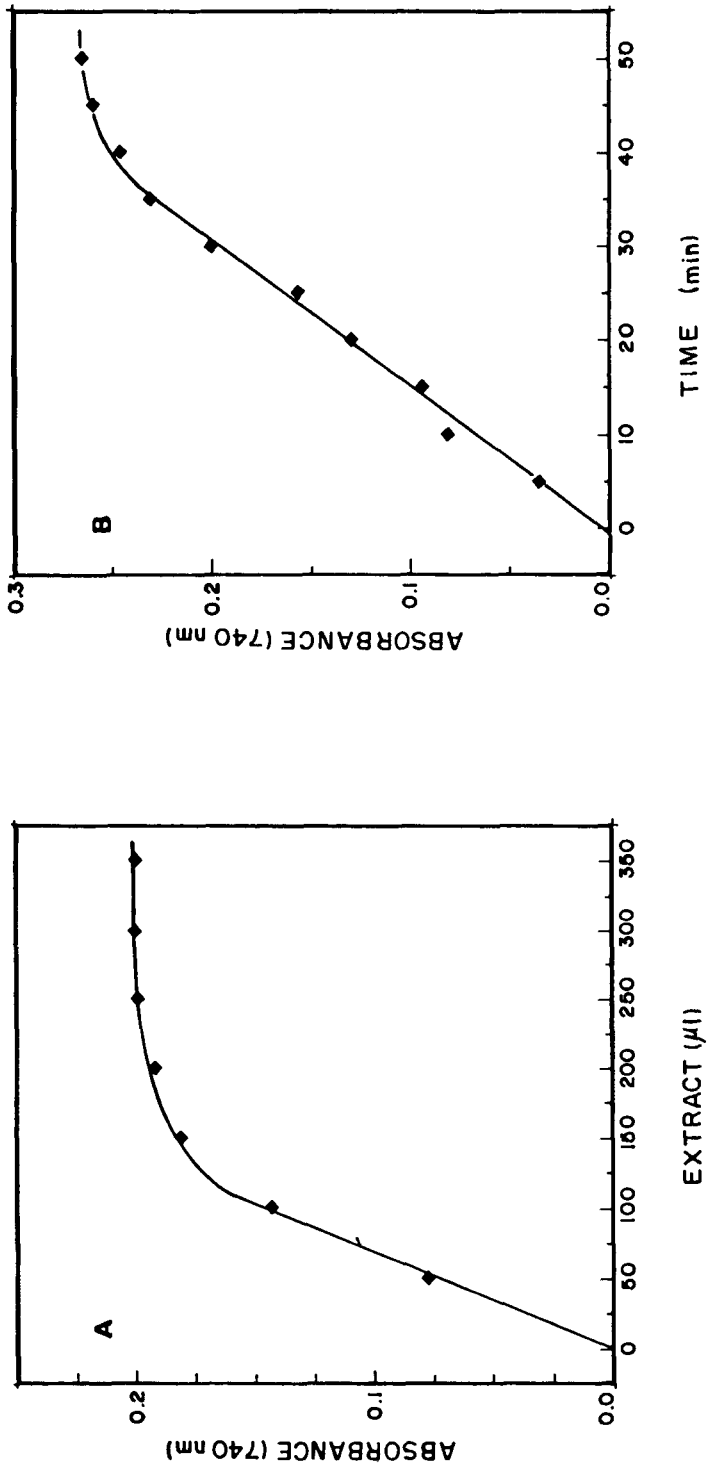


Fig. 1. Enzyme concentration effect on the rate reaction (A) and time course (B). For experiments in A the time was 10 min. The enzyme concentration in B was 200 μL of crude extract.

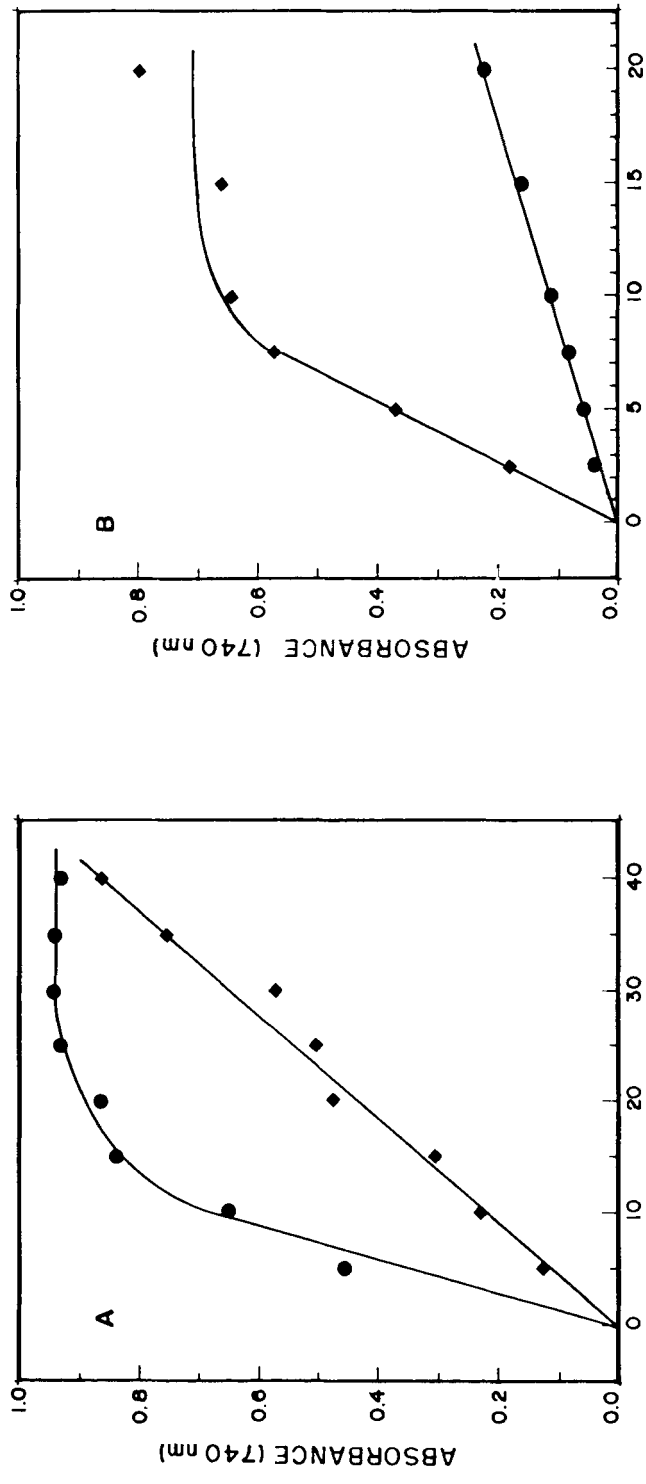


Fig. 2. A. Activation effects on the enzyme activity at time zero (◆) and after 24 h (●), the samples were kept at −20°C. B. Enzyme activity with (◆) and without (●), activators after 10 d to store at −20°C.

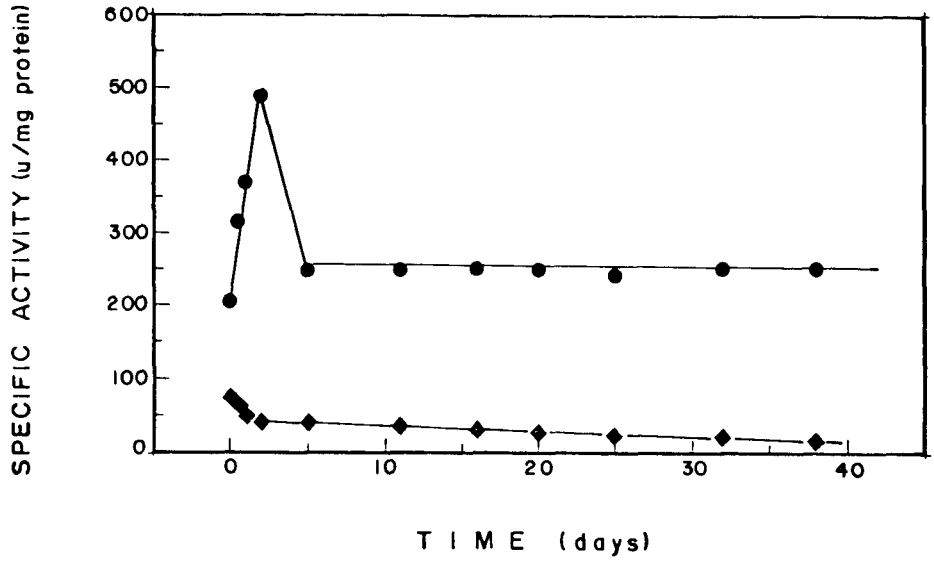


Fig. 3. Enzyme activity with (●), and without (◆), activators after 10 d stored for different periods of time at −20°C.

Table 1
Proteolytic Activity Purification from *A. fourcroydes*

| Step | Total protein, mg | Total activity, u | Specific activity, u/mg prot | Times of purification | Yield, % |
|---|---|---|---|---|---|
| pH 6.0 | | | | | |
| Crude extract | 144 | 94176 | 654 | — | 100 |
| 33% ethanol | 6 | 90726 | 15042 | 23 | 96 |
| G-200 | 4 | 88452 | 20915 | 32 | 93.9 |
| pH 9.6 | 154.8 | 140400 | 907 | — | 100 |
| | 10 | 136315 | 13605 | 15 | 97 |
| | 2.2 | 136050 | 62695 | 69 | 96.9 |

## pH-activity Profile

To determine the optimal pH for the semipurified extracts, a scrutiny of proteolytic activity in a pH range from 5–11 was carried out. As shown in Fig. 4, two distinct activity peaks occurred, one at pH 6.0 and the other at pH 9.8.

## Thermal Stability

The thermostability of these proteases was examined by preincubating the enzyme solutions for 30 min in the temperature range 30–70°C

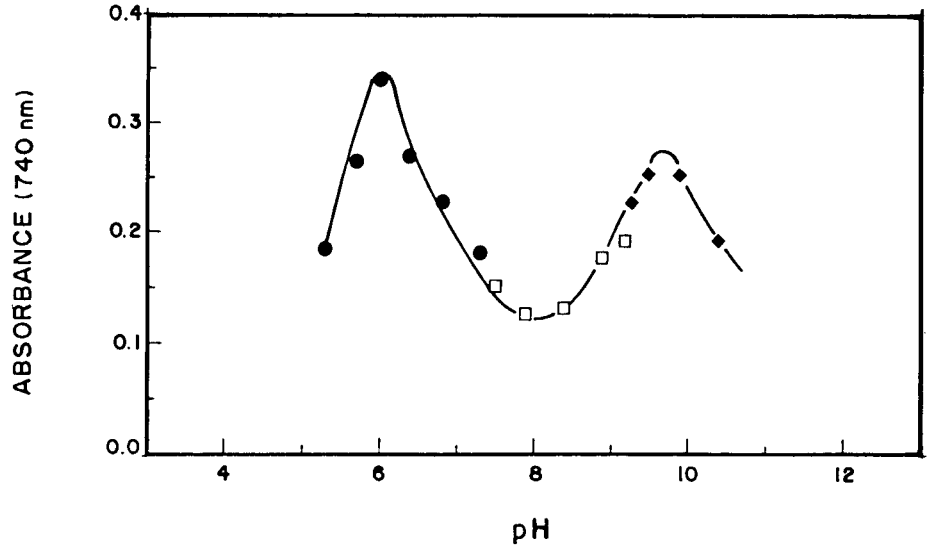


Fig. 4. pH profile of peak I. Citrate-phosphate for pH 5–7.4 (●); Tris-HCl for pH 7.4–9.2 (□), and carbonate-bicarbonate for pH 9.2–10.5 (◆).

(Fig. 5A). The thermostability of the peaks differed, with peak I being more stable than peak II. In both cases, peak stability was reduced at higher temperatures. Also, we incubated the enzyme from both peaks at 45°C for different periods of time. Enzyme activities were measured at pH 6 and 9.8. The proteolytic activity at pH 6 was more stable than the activity at pH 9.8 (Fig. 5B).

### Molecular Weight

Using the same gel filtration column, the molecular weights of the proteases, estimated from their elutions volumes, were 14,500 and 12,000 d (Fig. 6).

## DISCUSSION

We have semipurified two proteolytic activities from *Agave fourcroydes* juice. Both enzymes differ in molecular weight (Fig. 6). Peak I has a mol w of 14,500 d, and peak II 12,000 d. The optimum pH for peak I activity was 9.8 and for peak II 6.0. These data suggest that peak II may be a cystein-dependent protease and that peak I may be a serine protease. To test this possibility, both enzymes were tested in the presence of *p*-chloromercuri-benzoic acid and $CaCl_2$. The results are shown in Table 2. Both enzymes are cystein dependent, especially peak I, which is also calcium-dependent. In the presence of calcium ions, peak I activity increased 600%, whereas peak II activity was unmodified. These properties are consistent with

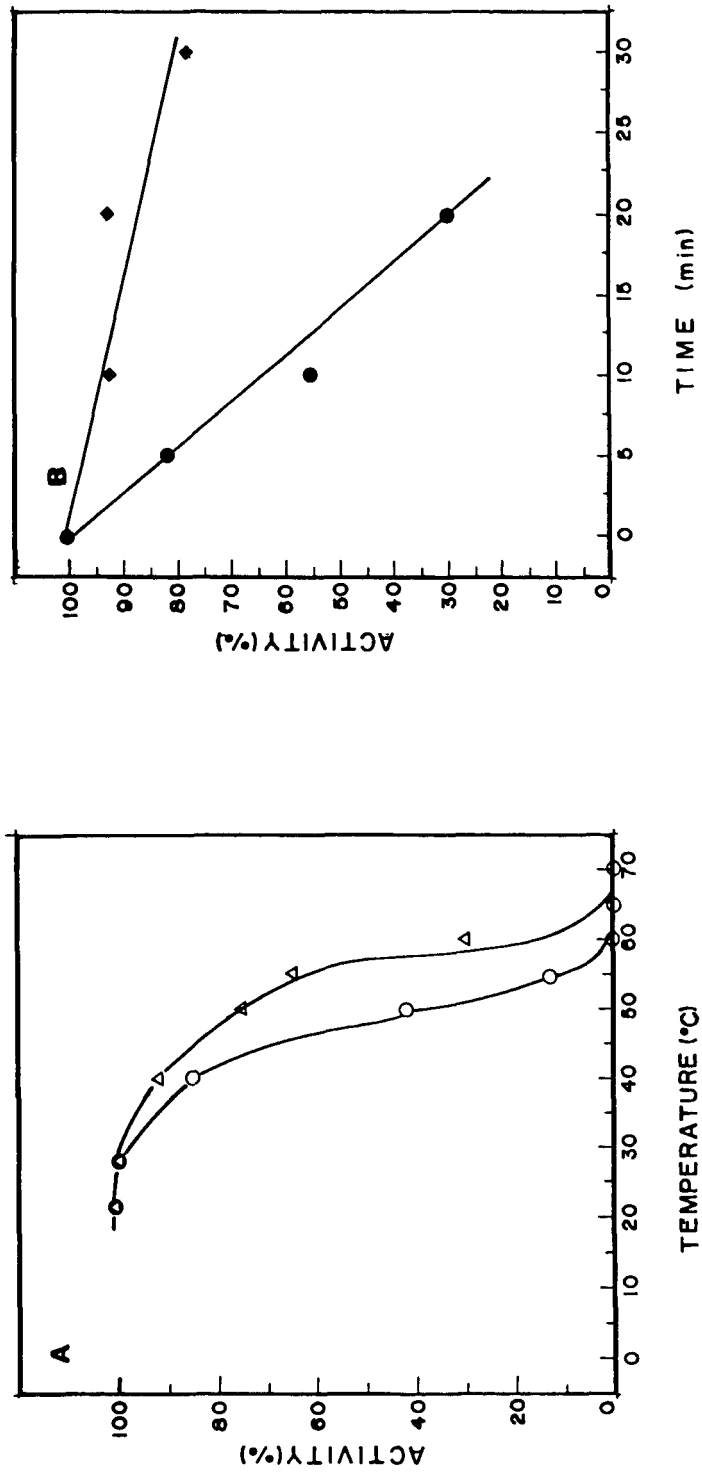


Fig. 5. A. Thermal stability of peak I (△), and peak II (○), from the G-200 column elution. B. Temperature effect on the protease activity from peak I measured at pH 6 (◆), and pH 9.8 (●). The samples were incubated at 45°C for the indicated time and then measured at the different pHs.

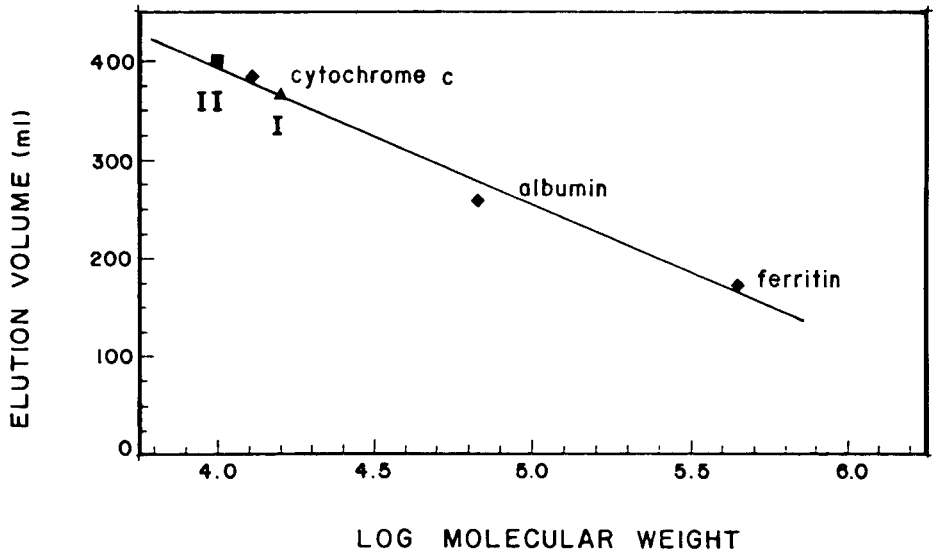


Fig. 6. Molecular weight determination. The samples were applied to a G-200 Sephadex column (90×2.6 cm); cytochrome c 12,400 daltons; albumin 66,000 daltons; and ferritin 443,000 daltons.

Table 2
Inhibitors' Effects on Proteolytic Activity from *A. fourcroydes*

| Inhibitor | Activity, pH 6 | % over the control pH 9.8 |
|---|---|---|
| pHMB | 57.3 | 8.1 |
| $CaCl_2$ | 89.0 | 610 |

the conclusion that peak I is a serine protease and that peak II is a cystein protease.

The pH optima of these enzymes differ from both the *A. sisalane* protease, agavain (2), and *A. americana* protease (4) in pH optima. However, peak II (pH 6) resembles the *A. americana* protease in that it does not require metal ions for optimal activity.

A new round of purifications was performed and the proteolytic activity was measured at both pH 6 and pH 9.8 (Table 1). Peak I (pH 9.8) forms 60% of the total proteolitic activity, suggesting that potentially it is the most interesting preparation for industrial processes.

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