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Comparison of Methods for Determining Myrosinase Activity

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Four assays for plant myrosinase (EC 3.2.3.1) were compared for linearity, sensitivity, reproducibility, and suitability for routine analyses. Methods: (i) pH-stat assay (pHSA); (ii) spectrophotometric coupled enzyme assay (SCEA); (iii) direct spectrophotometric assay (DSA); (iv) a new polarographic coupled assay (PCA) involving glucose oxidase and catalase, which measures the rate of glucose release as O_2 uptake during substrate hydrolysis. PCA and pHSA showed comparable activities and were linear with increasing amounts of purified enzyme up to 10 μg. As originally proposed, SCEA showed complete nonlinearity due both to the presence of ascorbate as myrosinase activator and to the low concentrations of Mg^{2+} and hexokinase–glucose 6-phosphate dehydrogenase. None of these methods appear suitable for routine work for different reasons. On the contrary, although DSA gave the expected lower activity compared to pHSA due to the suboptimum substrate concentration, it appears the most suitable for routine analyses given its simplicity and reliability.

Meals of rapeseed and other cruciferous seeds have high protein content and a well-balanced amino acid composition (Van Etten et al., 1965). However, the presence of large amounts of glucosinolates (thioglucosides) limits their use as feed, especially for meals produced with varieties not improved for this characteristic.

Glucosinolates are not deleterious in themselves; however, in the presence of myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) they degrade rapidly to give glucose, bisulfate, and an aglucon, which can undergo a spontaneous Lössen rearrangement to produce goitrogenic isothiocyanates or, via a protonation mechanism at low pH, toxic nitriles (Gil and MacLeod, 1980) as outlined in Scheme I.

The enzyme myrosinase seems to occur in all plants that contain glucosinolates (Ettlinger and Kjaer, 1968) even though enzymes with myrosinase activity have also been detected in some microorganisms, insects, and mammals (Björkman, 1976). Myrosinase appears to be an important enzyme, mostly for the biological and technological implications in food and feed quality and the safety of cruciferous material. For studies in this field it is evident that a dependable, rapid, and inexpensive assay to measure myrosinase activity is essential.

Over the last decade, numerous publications have described indirect and direct techniques for assaying myrosinase from several sources. The direct methods, viz. titration of released acid with alkali using pH-stat apparatus (Tookey and Wolff, 1970; Björkman and Lönnerdal, 1973), spectrophotometric measurements of the decrease in absorbance at 227 nm during sinigrin disappearance (Schwimmer, 1961; Gil and MacLeod, 1980; Palmieri et al., 1982), and recently a spectrophotometric coupled assay that measures the released glucose via hexokinase–glucose 6-phosphate dehydrogenase (Wilkinson et al., 1984a,b)

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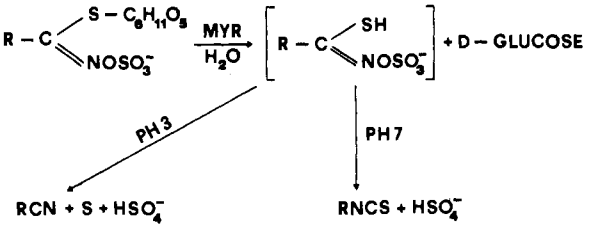
Scheme I



appear to be better than the indirect ones and are in theory all equally efficient, simple to use, and suitable for kinetic analyses as they allow one to determine myrosinase activity continuously. However, to our knowledge the relative advantages and disadvantages of the cited methods have never been extensively and objectively examined, especially in view of their utilization in breeding programs and in comparative studies of different plant tissues and cruciferous species.

This paper is a continuation of our work on cruciferous oil-bearing seeds (Palmieri et al., 1982, 1986; Iori et al., 1983). These studies prompted us to find other convenient methods for routine myrosinase determination that overcome the potential differences between enzyme sources and/or isoenzymes that could affect activity measurement. Therefore, the purpose of this study was to find a simple, dependable, and inexpensive assay that can rapidly analyze large numbers of crude or partially purified myrosinase samples. To this aim, an additional new polarographic assay, not previously used for this purpose, was tested. The present paper is a comparative study of four methods and also examines the effect of ascorbic acid.

MATERIALS AND METHODS

Materials. Commercial varieties of rapeseeds, *Brassica napus* L. cv. Jet Neuf (low erucic acid) and cv. Jade (low erucic acid and glucosinolate contents), were obtained from Ringot (Lille, France) and Norddeutsche Pflanzenzücht (Hohenliet, W. Germany) respectively. White mustard

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seeds, *Sinapis alba* L. cv. Albatros, were supplied by S. I. S. Foraggera (Bologna, Italy). Sinigrin, sulfate-free hexokinase type F-300, sulfate-free glucose 6-phosphate dehydrogenase type XV, standard glucose solution, ATP, and NADP were supplied by Sigma Chemical Co. (St. Louis, MO); glucose oxidase and catalase were from Fluka (Buchs, Switzerland). The other reagents were of analytical grade.

Preparation of Myrosinase Extracts. The defatted meals were homogenized with distilled water (1:20, w/v) with an Ultra-Turrax Model TP 18-10 IKA-Werk (Staufen, W. Germany). The insoluble material was removed by centrifugation at 17700*g* for 20 min; the supernatants were filtered with filter paper and dialyzed thoroughly against distilled water; the precipitated material was removed by centrifugation. The volume of the myrosinase extracts was measured and then analyzed for myrosinase activity and protein content. All steps in the procedure were performed at 4 °C.

Myrosinase Purification. The enzyme extracted from white mustard was purified according to our previously reported procedure (Palmieri et al., 1986), lyophilized, and stored in small amounts at −20 °C. The myrosinase was dialyzed against triply quartz-distilled water before use in activity tests.

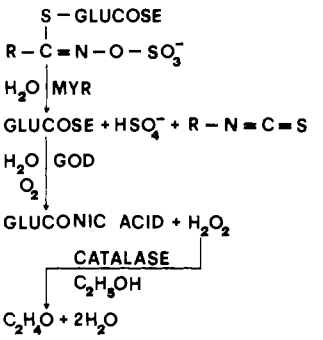
Direct Spectrophotometric Assay (DSA). The activity was determined by measuring the decomposition of the sinigrin substrate by following the decrease in absorbance at 227 nm with quartz cells with a 0.5-cm path length in a Model 219 Cary recording spectrophotometer. To compare this method with the other assays, the standard reaction mixtures and the conditions were slightly modified from those previously described (Palmieri et al., 1982) and were as follows. The temperature was 30 °C, although the best signal-to-noise ratio has been obtained at 37 °C, which is particularly advantageous for crude extract analyses. The standard reaction mixture contained 0.5 mM sinigrin, 33 mM phosphate buffer (pH 6.5), 100 μL of appropriately diluted myrosinase, and 0.5 or 1.0 mM ascorbate when present, in a total volume of 1.5 mL.

Spectrophotometric Coupled Enzyme Assay (SCEA). The conditions and the standard mixture were essentially those reported by Wilkinson et al. (1984a): 30 mM Mes buffer (pH 6.5), 3 mM $MgCl_2$, 0.55 mM ATP, 0.72 mM NADP, 5 mM sinigrin, 100 μL of appropriately diluted myrosinase, 0.56 U hexokinase, 0.35 U glucose 6-phosphate dehydrogenase, and where indicated, 0.5 or 1.0 mM ascorbate, in a total volume of 1 mL. The activity was measured at 30 °C, observing the increase of absorbance at 340 nm during the formation of NADPH from NADP with 1-cm path length quartz cells in a Model 219 Cary recording spectrophotometer.

pH-Stat Assay (pHSA). The composition of the standard mixture was 5 mM sinigrin, 80 mM NaCl, and 50 μL of appropriately diluted myrosinase and 0.5 or 1.0 mM ascorbate when present. The total assay volume before titration was 5 mL, and all reagents, myrosinase included, were prepared in triply quartz-distilled water. The pH was adjusted to 6.5. Myrosinase activity was determined by measuring the acid release rate by titrating with 1 mM NaOH with a E 457 Metrohom dispenser (Herisau, Switzerland), connected to a 5-mL EA 928-5 buret and a Model PHM63 Radiometer pH-meter (Copenhagen), maintained at pH 6.5. During the reaction the solution was kept at 30 °C in a thermostatically controlled cell and gently stirred magnetically.

Polarographic Coupled Assay (PCA). The myrosinase activity test was performed by determining the rate of oxygen uptake with a Clark electrode in a Gilson K-IC Oxygraph, Medical Electronics (Middleton, WI). An excess of glucose oxidase was used to oxidize quickly the released glucose produced from the glucosinolate hydrolysis catalyzed by myrosinase according to Scheme II. The glucose oxidase solution had the same composition as that used in a previous study for determining glucosinolate content in cruciferous material (Iori et al., 1983). The reaction mixture for determining myrosinase activity contained the glucose oxidase solution, 5 mM sinigrin, and appropriately diluted myrosinase and 0.5 or 1.0 mM ascorbate when present. During the reaction it was thermostatically maintained at 30 °C and gently stirred magnetically in a total volume of 1 mL. Before use, the glucose oxidase solution was maintained at 30 °C for at least 2 h with occasional stirring.

Scheme II



Protein Determination. Protein concentration was measured by the method described by Bradford (1976) using the Bio-Rad protein assay.

Calculation of Myrosinase Activity. *DSA.* The initial rate of decrease of absorbance at 227 nm produced by sinigrin hydrolysis was determined from E_{227nm} (6784 M^{-1} cm^{-1}) as previously reported (Palmieri et al., 1982). One unit of activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 nmol min^{-1}.

SCEA. Myrosinase activity was calculated as the rate of absorbance increase at 340 nm after preequilibration of the reaction mixture for few minutes (because a lag was observed before the linear change in absorbance) during the formation of NADPH from NADP. Initial velocities of sinigrin hydrolysis were determined from the well-established E_{340nm} (6220 M^{-1} cm^{-1}) for NADPH.

pHSA. As reported by Croft (1979) and Tookey et al. (1980), 1 mol of glucosinolate breaks down to yield 1 equiv of hydrogen sulfate ion. Enzymatic activity was measured by determining the nanomoles of NaOH required to neutralize the acid released every 30 s for a total observation time of 5 min. The sinigrin hydrolysis rate was determined by calculating the slope of the straight line obtained by plotting nanomoles of NaOH against time in minutes by the least-squares procedure.

PCA. It is well established that 1 mol of glucose is released from 1 mol of glucosinolate; therefore, myrosinase activity was calculated from the rate of glucose released from sinigrin measured as O_2 uptake on the basis of Scheme II. After the oxygraph was calibrated with a standard glucose solution, we chose a glucose amount, G (μg), as a reference point in the range of instrument linearity that corresponds to a signal calculated as the average of five determinations

$$\text{nmol min}^{-1} = \frac{D \times G \times 1000}{N \times 180}$$

where D is the amplitude of the signal corresponding to the glucose released from sinigrin in 1 min and N is that

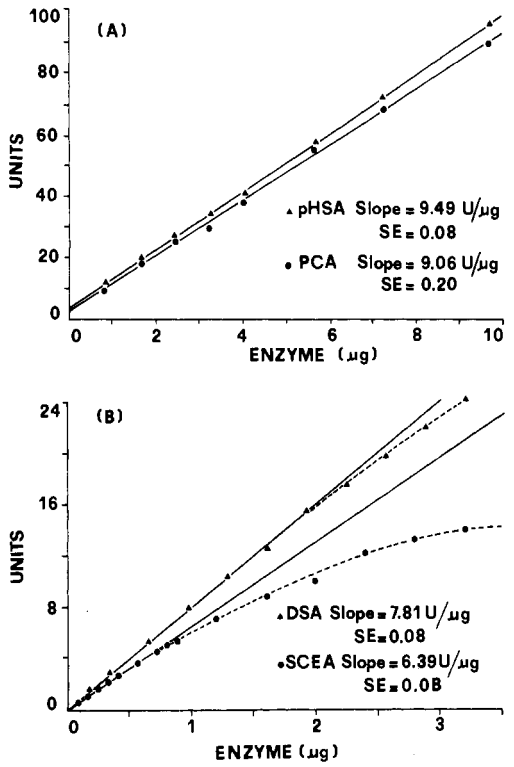


Figure 1. Enzyme concentration dependence of myrosinase activity: (A) pHSA and PCA; (B) DSA and SCEA. The specific activities calculated from the slope of each curve in the range of linearity are also reported.

of the reference glucose amount.

RESULTS AND DISCUSSION

In the present study the specific activity data and the ranges of linearity are not comparable to those in the literature because the myrosinase had better purity and was used after lyophilization.

As reported by other workers (Wilkinson et al., 1984a) pHSA should be considered as the reference method, although in its use care must be taken in choosing the alkali concentration to achieve measurable reaction rates.

Assay Linearity, Sensitivity, and Reproducibility. Figure 1 shows the effect of enzyme concentration on the activity of the myrosinase-catalyzed reaction of sinigrin hydrolysis as measured by the four methods. pHSA and PCA show good linearity in a very wide range of enzyme concentration (Figure 1A), whereas for DSA and SCEA the range of linearity is much narrower (Figure 1B). In fact, the activity measured with SCEA is linearly proportional to the amount of myrosinase only until 0.8 μg; that obtained by DSA appears to be linear until almost 2 μg of enzyme. The values of specific activity are reported next to the curves in Figure 1. These were obtained by calculating the slope of each curve in its range of linearity. From these data it appears evident that the activity measured by PCA is the closest to that determined by pHSA, and the slight difference does not appear significantly (P = 0.05, df 12). On the other hand, DSA and SCEA show activities that are about 18% and 33% smaller than pHSA, respectively. The difference recorded for DSA was expected because of the lower substrate concentration (0.5 mM), which is near the maximum for a cell with a 0.5-cm path length. However, this substrate concentration is about 3 times higher than the apparent K_m value [0.156 mM (Palmieri et al., 1982), 0.17 mM (Björkman and Lönnerdal, 1973)] and therefore high enough to determine the initial velocity of the hydrolysis reaction for steady-state kinetic studies of enzyme. On the other hand, the low activity obtained by SCEA is surprising, since in this case the substrate concentration (5 mM) was the same as that used for pHSA and PCA. This loss of activity presumably depends on the low global first-order rate constant of the second part of reaction that transforms glucose to 6-phosphogluconate, which is catalyzed by hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PDH) with a consequent glucose accumulation during the reaction.

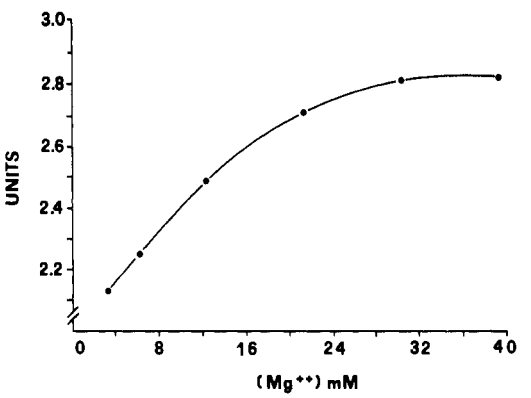


Figure 2. Effect of Mg^{2+} concentration on myrosinase activity determined by SCEA. The amount of myrosinase used was 0.32 μg.

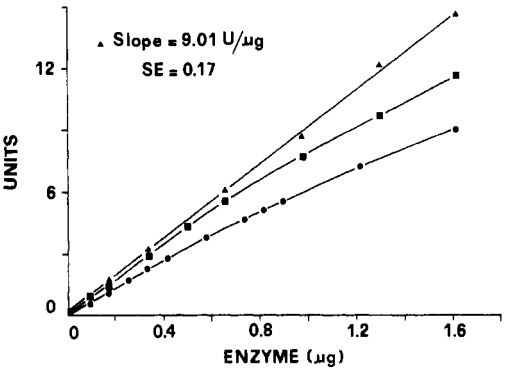


Figure 3. Enzyme concentration dependence of myrosinase activity determined by SCEA in the presence of different amounts of Mg^{2+}, HK, and G6PDH: (●) Mg^{2+} 3 mM, HK 0.56 U, G6PDH 0.35 U; (■) Mg^{2+} 30 mM, HK 0.56 U, G6PDH 0.35 U; (▲) Mg^{2+} 30 mM, HK 5.6 U, G6PDH 3.5 U.

We can now consider the reasons for the lower activity obtained by SCEA. The results reported in Figures 2 and 3 demonstrate that the concentrations of $MgCl_2$ and coupled enzymes (HK, G6PDH) proposed by previous workers (Wilkinson et al., 1984a) are insufficient to obtain a linearity and sensitivity comparable to the other assays. In fact, as Figure 3 shows, when the concentrations of Mg^{2+} ion and coupled enzymes (HK + G6PDH) are increased, the resulting activities become proportional to the myrosinase concentration used, thus restoring the linear response of the assay. In these conditions the specific activity of myrosinase determined by SCEA was not significantly different from that measured by pHSA and PCA (P = 0.05, df 10).

Table I shows the specific myrosinase activity in the partially purified extracts of two varieties of rapeseed with high and low glucosinolate contents (Jet Neuf and Jade, respectively) and from white mustard seed determined by the four methods of analysis compared. In all cases the activity measured by pHSA appears higher than for the other assays, thus providing a good sensitivity for analyzing extracts with low activities. If one considers the data

Table I. Myrosinase Specific Activity (U/mg) Measured in Extracts of One White Mustard and Two Rapeseed Varieties[a]

	DSA	SCEA	pHSA	PCA
rapeseed cv. Jet Neuf	229 ± 2	230 ± 2	264 ± 2[b]	nd[c]
rapeseed cv. Jade	139 ± 1	142 ± 1	160 ± 3[b]	nd[c]
white mustard cv. Albatros	3580 ± 15	2565 ± 15	4155 ± 35	4140 ± 30

[a] Each value is the average ± SD of four replications. [b] Determined with 400 μL of extract. [c] nd = not detectable.

obtained with DSA and SCEA, those for rapeseed are in good agreement, while for those for mustard extract, in which the activity is much higher, SCEA shows a considerable loss in sensitivity, thus confirming the results achieved for pure myrosinase. The polarographic method, in the conditions used, proved to be unsuitable for measuring extracts with low activities because the maximum volume of sample that can be analyzed does not afford an accettable, reproducible signal.

Effect of Ascorbate. It does not appear convenient to use ascorbate as an activator in the assay mixture to measure the activity of myrosinases from different sources as been proposed for SCEA (Wilkinson et al., 1984a,b). In fact, Björkman and Lönnerdal (1973) demonstrated that activation with ascorbate strongly depends on the plant species, and moreover, varieties within the species also differ in this property. In addition, contrary to that reported in a previous paper (Wilkinson et al., 1984a) in which pHSA and SCEA showed the same activity trend, the specific activities that we measured for the four assays in the presence of 0.5 mM ascorbate as activator differed remarkably (Figure 4A,B). These differences persisted when the ascorbate concentration was increased to 1.0 mM (not shown). The methods considered are probably inadequate when used in the presence of this activator. For example, in the case of PCA, the catalase used in the assay mixture could be at least in part inhibited by ascorbate (Orr, 1967), which can also act as a scavenger of the free radicals involved in the reaction chain of glucose oxidation. The reduced linearity observed for SCEA (Figure 1B) becomes even more reduced when ascorbate is used (Figure 4B), thus making it difficult to choose the assay conditions and, in this case, to calculate the slope for the specific activities. The use of ascorbate with DSA and pHSA, within the known limits of concentration, seems more feasible than the other methods, even if from the comparison of their activity data it appears that the value obtained with DSA is significantly higher. In conclusion, taking in account that these last results are contradictory and that the influence of chemical and physical conditions on the activation mechanism of myrosinase by ascorbate is still poorly understood, we are convinced that the use of this activator in myrosinase assays should be avoided, particularly when the reaction mixture is complex, such as PCA and SCEA.

Conclusions. In addition to the results concerning the accuracy, precision, and applicability of the enzyme concentration range in the compared methods, some considerations on their suitability seem in order both for routine analyses and for enzyme purification and characterization studies. Although pHSA is the most reliable assay, it has a number of drawbacks when used in routine work. Its main disadvantage is without doubt the need to dialyze thoroughly all buffered myrosinase extracts before analysis, which greatly increases the analysis time. Although PCA had a wide range of linearity in the condition used, it requires dialysis to remove glucose from the samples before

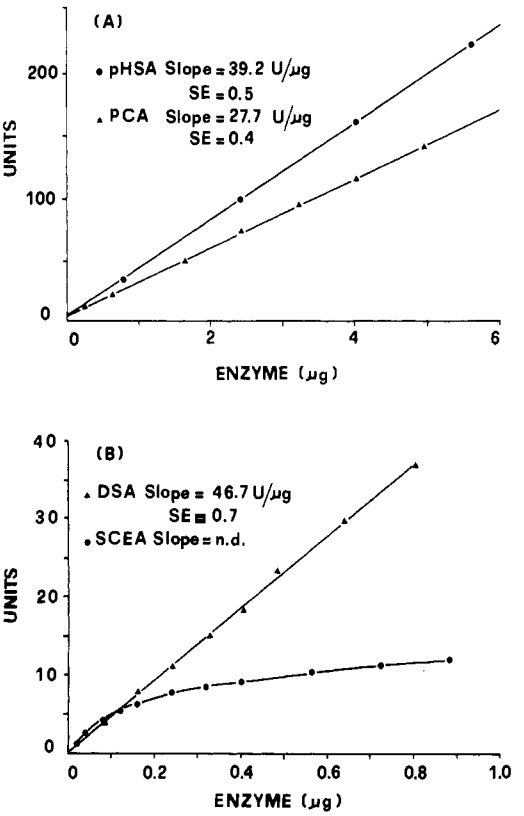


Figure 4. Enzyme concentration dependence of myrosinase activity in the presence of 0.5 mM ascorbate as enzyme activator: (A) pHSA and PCA; (B) DSA and SCEA. The specific activities calculated from the slope of each curve in the range of linearity are also reported.

analysis and shows poor sensitivity and reproducibility when used in analyses of extracts with low activity. This is why we feel polarography cannot be used for routine myrosinase activity measurements in rapeseed extracts, even if it is a useful alternative technique to measure the activity during the enzyme purification process starting from white mustard. In theory, SCEA appears to have good characteristics for use in routine work. However, this method, as proposed by the authors, i.e. in the presence of ascorbate as activator (Wilkinson et al., 1984a), gave results in disagreement with the other assays tested. In addition, these results are clearly affected by the nonlinearity of the method, as shown in Figure 4B. This aspect was pointed out, but not investigated, by Wilkinson et al. (1984b) in a paper in which myrosinase was studied in several cruciferous vegetables. On the other hand, we think that SCEA would be suitable for routine analyses of myrosinase extracts if it is employed without ascorbate and if the concentrations of Mg^{2+} ion and coupled enzymes (HK–G6PDH) are increased in the reaction mixture at least 10 times with respect to the original method. We feel it our duty, however, to point out that the proposed variations involve an increase in the analysis cost, which is already rather high compared to the other assays. With regard to the applicability of this assay to the enzyme characterization studies, it shows some disadvantages mainly attributable to the particular molecular properties of HK and G6PDH, enzymes that are much more delicate and sensible to variations in pH and temperature than myrosinase. For kinetic studies we believe that the DSA, as shown in a previous work (Palmieri et al., 1982), remains the most suitable assay, for both its simplicity of execution and the reliability of its results, especially when a high-performance spectrophotometer in good working condition

is available. In fact, the best feature of this technique is the possibility of observing the enzyme reaction rate continuously and directly, with the advantage that one can use a wide range of temperatures, pHs, and possibly, within certain limits of concentration, some effector compounds. In addition, we think that DSA can also be useful in routine analyses of myrosinase extracts, even though the signal-to-noise ratio must be improved when a suitable spectrophotometer is not available by decreasing the high absorbance values of the extracts by rapid dialysis or fast gel filtration systems.

Registry No. Myrosinase, 9025-38-1.

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Production of Protease from Cell Cultures of Common Milkweed (*Asclepias syriaca* L.)

Dietrich Knorr* and Susanne M. Miazga

Protein content and proteolytic activity of common milkweed (*Asclepias syriaca*) cell cultures and culture media were investigated on batch cultures of freely suspended as well as calcium alginate gel or chitosan gel entrapped cells. Maximum protein content of freely suspended cells and culture media was reached at a culture age of 28 days. The protein concentration from chitosan entrapped cells was higher than that of the calcium alginate entrapped cells during 96 h of experiments. Proteolytic activity was detected in the cell homogenates and in the culture media from freely suspended and gel-entrapped milkweed cells.

Proteolytic enzymes are used extensively in various industries. Applications in the processing of food include oriental fermentations, cheese coagulation and ripening, modification of functional properties of proteins, bread making, and chill proofing of beverages (Fox and Morrissey, 1980; Liener, 1974; Löffler, 1986; Peterson and Johnson, 1978; Schwimmer, 1981a). The leather industry utilizes them to prepare hides for tanning, the textile industry to reduce shrinkage of fibers, and the laundry and dry cleaning industry to remove stains (Ward, 1985). Medical and therapeutic uses of enzymes, particularly in the treatment of gastric bezoars (Graham, 1981) and as a nonsurgical alternative in the treatment of herniated lumbar intervertebral discs (Clark and Witherspoon, 1983; Gunby, 1983; Klausner, 1983) further illustrate the wide range of applications for proteolytic enzymes.

Cysteine proteinases (thiol proteinases) that are commonly used include papain (EC 3.4.22.2) and chymopapain (EC 3.4.22.6) from papayas, ficin (EC 3.4.22.3) from figs, and bromelain (EC 3.4.22.4) from pineapples (Peterson and Johnson, 1978; Schwimmer, 1981b; Ward, 1985; Wolnak, 1980).

The proteolytic activity of the latex of *Asclepias speciosa* Torr. was identified in the 1940s (Winnick et al., 1940), and asclepain (EC 3.4.22.7) was first crystallized from the pressed juice of roots of the common milkweed (*Asclepias syriaca* L.) by Carpenter and Lovelace (1943). Comparison of amino acid sequences of the asclepains isolated from *A. syriaca* L. latex (Brockbank and Lynn, 1979) with those of papain reveal extensive homologies, suggesting that although milkweed and papaya plants are unrelated, perhaps an ancestral gene for the enzyme is shared (Lynn et al., 1980).

Proteolytic enzymes are present in tissue cultures of papaya and pineapple (Medora et al., 1973; Apte et al., 1979; Mathews et al., 1976). Milkweed has been cultured (Biesboer, 1983) and regenerated (Singh, 1984) in vitro; however, proteolytic activity in *Asclepiadaceae* plant cell cultures has not been investigated.

The objectives of this study were to examine protein content and proteolytic activity in culture media and cell homogenates of immobilized and freely suspended *A.*

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