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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Received for review July 7, 1986. Accepted February 9, 1987. This work was performed as a part of the Project for Yield and Quality Improvement of Oleagineous Plants supported by the Italian Ministry of Agriculture. Part of the results of this study was presented at the E.E.C. Workshop on Glucosinolates in Rapeseeds, Oct 1–3, 1986, Gembloux, Belgium.

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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syriaca L. cells, as a potential means for proteolytic enzyme production.

MATERIALS AND METHODS

Seeds from common milkweed (*A. syriaca* L.) collected in northern Delaware, in late October, were manually removed from the follicles and used to initiate tissue cultures. Callus cultures were initiated on a basal Murashige and Skoog (1962) medium (MS) supplemented with 100 mg of myoinositol, 400 mg of casein hydrolyzate, 1.0 mg of kinetin, 1.0 mg of α-NAA, and 6.0 g of Difco-Bacto agar/L of medium. An agar-free MS medium described above with a modified growth hormone concentration of 1.5 mg of kinetin and 1.5 mg of α-NAA/L was used in initiating and maintaining suspension cultures.

Immobilization of *A. syriaca* L. cells was carried out with alginic acid, sodium salt, type IV (Sigma Chemical Co., St. Louis, MO), and with commercially available chitosan (Bioshell Inc., Albany, OR) as described earlier (Knorr and Teutonico, 1986).

A. syriaca L. seeds were disinfested with 70% ethanol for 2 min and 0.5% sodium hypochlorite for 15 min, rinsed with sterile distilled water, and then placed in sterile 100 × 15 mm Petri dishes containing moist, sterile, germination paper. The dishes were covered, wrapped in aluminum foil, and incubated at 25 °C. After 6 days, germination was nearly 100%. When the seedlings were approximately 30 mm in length, segments of the hypocotyls and primary leaves were treated with 70% ethanol for 2 min and rinsed with sterile distilled water. Each explant was placed in a well of a 6-well Falcon culture plate (Becton Dickinson Laboratories, Oxnard, CA) containing 2.5 mL of the callus medium and incubated at 25 °C under constant illumination of approximately 25 400 lx. After 4 weeks, callus cultures were transferred to 50-mL Erlenmeyer flasks containing 20 mL of gelled growth medium and, after an additional 4 weeks, were transferred to 125-mL culture flasks containing 35 mL of gelled medium. For initiation of suspension cultures, approximately 1.5–2.0-g portions of hypocotyl callus were transferred into 125-mL Bellco culture flasks containing 25 mL of supplemented liquid MS medium, placed on a Controlled Environment Incubator Shaker (New Brunswick Scientific Co., Inc., Edison, NJ) at 20 °C, and agitated at 100 rpm under continuous illumination (nine high-output F24T12 cool white fluorescent lamps at minimum distance of 42.5 cm).

A cell density of 0.75×10^5 cells/mL determined in a Sedgwick-Rafter cell counter (Dodds and Roberts, 1982) established subculturing time intervals. Three subculturings were performed prior to initiating those cultures to be used for subsequent analyses. Mean fresh weights of harvested cells filtered through MiraCloth (Calbiochem, La Jolla, CA) were used in establishing growth curves.

Harvested cells of the suspension cultures were homogenized (Model 7605, Palo Laboratory Supplies) in 15 mL of either distilled water or 50 mM $CaCl_2$ solution. The cells were homogenized at high speed for 1 min and centrifuged (Beckman Model J-21B, type JA-20, fixed-angle rotor) at 5000*g* for 15 min at 4 °C.

Cell viability was measured by their mitochondrial reduction of 2,3,5-triphenyltetrazolium chloride after the method described by Towill and Mazur (1975).

Immobilization of *A. syriaca* L. cells was performed on 30-day-old suspension cultures following the procedure of Brodelius et al. (1979) for calcium alginate entrapment and that of Vorlop and Klein (1982) using 0.014 M acetic acid as a solvent for chitosan. Three grams fresh weight of *A. syriaca* L. cells were used per 100 mL of polyelectrolyte solution.

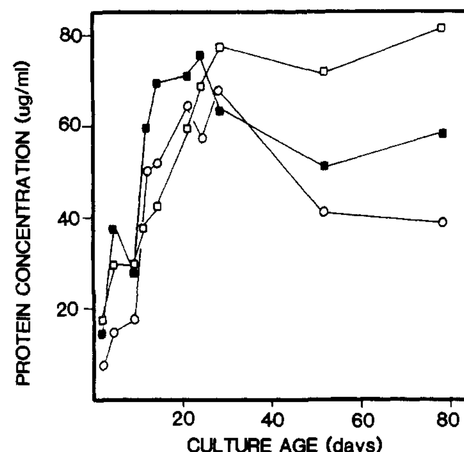

Figure 1. Protein concentration in culture medium and cell homogenates from suspension cultures of *A. syriaca* L. (n = 2–4): ○, culture medium; □, cell homogenate (in H_2O); ■, cell homogenate (in 50 mM $CaCl_2$).

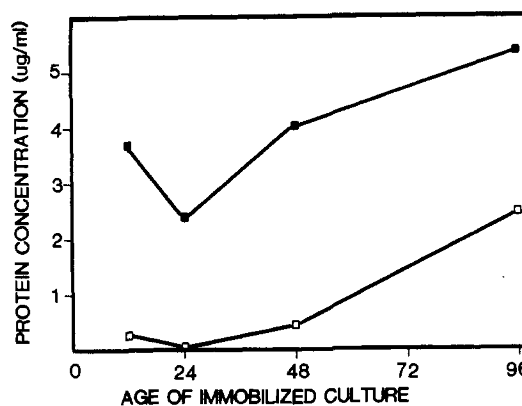

Figure 2. Protein concentration in culture medium from *A. syriaca* L. cell cultures immobilized in alginate (□) and chitosan (■) gels (n = 4).

Protein content of culture medium and cell homogenates was determined by the method of Bradford (1976). Culture medium was used as a blank in the standard preparations.

Protease kits (Bio-Rad Laboratories, Richmond, CA) were used for initial screening for proteolytic activity. Proteolytic activity was quantitated by the spectrophotometric measurement of caseinolytic activity of the protease (*Food Chemicals Codex*, 1966) at pH 6.0 and 40 °C for 60 min using Hammersten-type casein and papain reference standards (U.S. Biochemical Corp., Cleveland, OH) with an enzyme activity of 1422 μkat/mg of protein.

RESULTS AND DISCUSSION

Protein Concentration. Protein concentrations of media and homogenized cells obtained from suspension cultures of *A. syriaca* L. are given in Figure 1.

Maximum protein concentration in the culture media was reached after 28 days in suspension, and results of an analysis of variance showed significant ($P < 0.01$) interaction between protein content and the culture age. Results of a Dunnett's test, using day 2 (culture age) as a reference, showed first significant differences at day 12 ($P < 0.005$). Cell homogenates revealed rapidly increasing protein concentration with age until 28 days and a distinct effect of the method of homogenization on the data. Results of an analysis of variance indicated significant effects ($P < 0.01$) of culture age on protein concentration as well as a significant interaction ($P < 0.01$) between the two methods of homogenization.

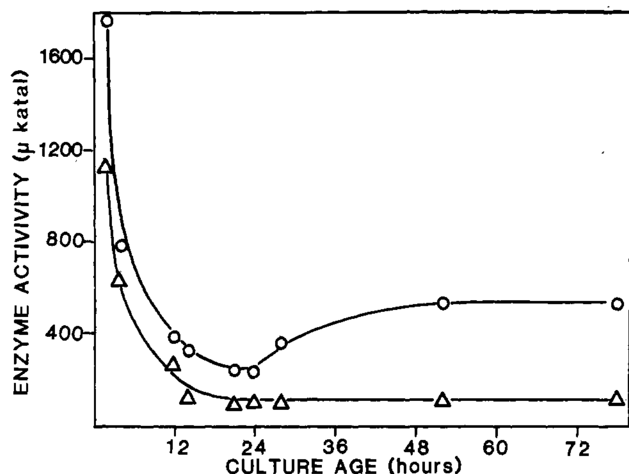

Figure 3. Proteolytic activity values of culture medium from freshly suspended cell cultures of *A. syriaca* L. (n = 4): O, μkat/mg of protein; Δ, μkat/g of fresh cell weight.

Protein concentration in media from immobilized cells entrapped in calcium alginate or chitosan gels is given in Figure 2. More protein was extracted from chitosan gel entrapped samples, reaching up to 5.4 μg/mL after 96 h, while significantly lower ($P < 0.05$) amounts (2.5 μg/mL) were obtained from calcium alginate gel entrapped samples. This could be due to a permeabilizing effect of chitosan on the plant cell membranes. Young et al. (1982) demonstrated that chitosan binds to polygalacturonate, a component of plant cell walls, and increases membrane permeability. In addition, the effect of chitosan as an immobilizing and concurrent permeabilizing agent has been shown recently with *Amaranthus tricolor* cells (Knorr and Teutonico, 1986).

Due to stability problems with chitosan gels, the duration of the immobilization experiments was not extended over 96 h; however, the data indicate the potential of chitosan gels for the development of continuous immobilized/permeabilized plant cell reactors. This is important since recent estimates suggest that technical advances, especially the development of continuous biocatalytic processes, can enable production of plant metabolites in a price range of \$20 to \$50/kg (Sahai and Knuth, 1985). This makes the development of immobilized plant cell reactors for biocatalytic processes an essential part of scale-up of plant cell culture systems (Knorr et al., 1985). Although the data in Figure 1 indicate the potential of cell homogenates for the continuous production of proteins from *A. syriaca*, advantages of immobilized plant cell reactors such as reuse of biocatalysts, high volumetric productivity, and improved product recovery and purification, as compared to traditional batch fermentation or to the labor-intensive production of crude papain and subsequent need for purification, can make continuous immobilized plant cell reactors effective tools for the production of high-purity protein.

Proteolytic Activity. Enzyme activity values are represented in Figure 3 and demonstrate a time-dependent decrease. Detection of proteolytic activity in the media of plant cell cultures suggests that the enzymes were released from the cell cytoplasm into the medium. Since proteolytic activity in cultures of common milkweed has not yet been reported, these data establish its presence in plant cell cultures of *A. syriaca* L. The decrease of activity can have various causes including the possibility of autolysis, the presence of endogenous protease inhibitors, or a feedback inhibition mechanism of the cultured plants that could be overcome when continuous cell reactors

Table I. Proteolytic Activity of Harvested Cell Homogenates from Suspension Cultures from *A. syriaca* L.

age of culture, days	activity[a] μkat/mg protein	activity[a] μkat/g fresh cell wt	age of culture, days	activity[a] μkat/mg protein	activity[a] μkat/g fresh cell wt
		In H_2O[b]			
2	1279	120	21	1046	20
4	832	79	24	601	17
8	641	102	28	1000	19
12	1009	48	52	1974	41
14	1591	28	78	752	21
		In $CaCl_2$[c]			
2	1628	129	21	1174	27
4	975	117	24	655	20
8	818	121	28	1835	29
12	683	31	52	1064	15
14	987	28	78	1456	28

[a] Means of four replications. [b] Homogenized in H_2O. [c] Homogenized in $CaCl_2$.

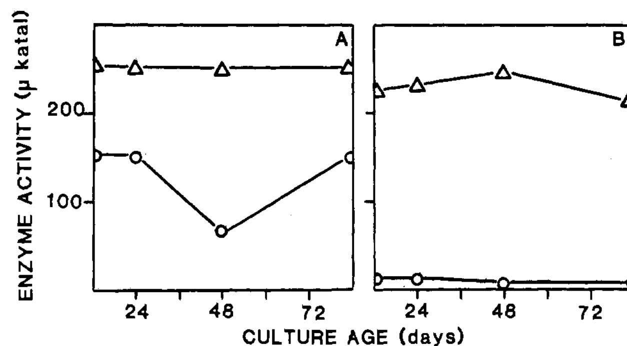

Figure 4. Proteolytic activity values of culture medium from *A. syriaca* L. cell cultures immobilized in alginate (A) and chitosan (B) gels (n = 4): O, μkat/mg of protein; Δ, μkat/g of fresh cell weight.

would be developed. Cell homogenates were also analyzed for proteolytic activity (Table I).

Calcium chloride or water was used as the homogenizing medium since Straus and Campbell (1963) have shown that calcium ions tend to release enzymes from possible binding sites in the walls of cultured plant cells. Overall, proteolytic activity of cell homogenates remained constant when based on protein although time-dependent fluctuations occurred. The activity based on fresh cell weight decreased until 14 days of culture age and then remained relatively constant. Since this does not parallel the growth of *A. syriaca* L. cells that reached a maximum at 28 days, additional factors must be involved. No effects of the homogenizing media could be observed.

Enzyme activity values detected in culture media of cultures immobilized in calcium alginate or chitosan gels are given in Figure 4. While proteinase activity of cells entrapped in calcium alginate was comparable to those immobilized in chitosan gels, activity based on protein was found to be substantially higher for calcium alginate than for chitosan gels. It is likely that this was due to additional release of nonproteinase protein from *A. syriaca* cells when chitosan was used as an immobilizing agent (Young et al., 1982). This possible effect is also evident from higher protein values for chitosan than for calcium alginate as given in Figure 2.

Overall, these results demonstrate that cultured *A. syriaca* L. cells were capable of producing and excreting protein(s) that exhibited proteinase activity under in vitro conditions. In addition, data on immobilization of cultured milkweed cells and their ability to produce proteolytic

substances suggest the potential for the development of plant cell reactors for the continuous production of such metabolites.

ACKNOWLEDGMENT

This work was partly supported by the National Sea Grant College Program under Grant No. NA83AA-D-00017, Project No. A/I-18. An award from the National Food Brokers Association to S.M.M. is also acknowledged.

Registry No. Protease, 9001-92-7.

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Received for review October 31, 1985. Revised manuscript received August 28, 1986. Accepted January 29, 1987.

Phospholipases C and D in Rice Grains

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Evidence for the relatively high activities of phospholipases C and D was found in rice grains. Both enzymes were present in both the outer and the inner layers of the brown rice. Kinetic analysis has shown that both enzymes acted independently, and no consecutive reaction chains have been found. Both enzymic reactions were of the first order, and no structural diffusion hindrance was found. The enzymes hydrolyzed not only different lecithins or cephalins but also phosphorylcholine, phosphatidic acids, and (*p*-nitrophenyl)phosphorylcholine. The specific rate constants with these substrates were similar but were higher with phospholipase D than with phospholipase C. The enzymes were still active at 70 °C. The activation energy of the phospholipase C was close to 41 000 J/mol.

Phospholipases (enzymes hydrolyzing lecithins and/or cephalins) are not identical with ordinary lipases. They were first found in snake, wasp, and scorpion venoms (Delezenne and Ledebt, 1910, 1911, 1912; Long and Penny, 1957). Phosholipases A and B hydrolyze lecithins or cephalins in positions 2 and 3, with the formation of the corresponding fatty acid and lysolecithin (lysocephalin). Phospholipase C hydrolyzes the phosphoric acid–glycerin bond on carbon 1 with the formation of phosphorylcholine, and phospholipase D attacks the phosphorylcholine bond with the release of free choline (Lowenstein, 1969). Except phospholipase D (by this definition), all phospholipases produce lyso compounds that are hemolytic and toxic once they reach the blood or brain (Delezenne and Ledebt, 1910, 1911, 1912; Contardi and Latzer, 1928; Zeller, 1952).

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Phospholipids play many important roles in metabolism. They interact with proteins and constitute an integral part of cell membranes, mitochondrial and microsomal cytochrome redox systems, or signal transfer cells. Many of the phospholipid functions are still unknown, especially in plants, and future work will evidently reveal much more about their importance.

Phospholipases have been demonstrated in plants, but except phospholipase D, they have not been studied as extensively as human phospholipases. Contardi and Ercoli (1933) have demonstrated phospholipases in rice bran.

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