Extraction and Partial Purification of Milk Coagulating Enzyme from *Cartamus tinctorius* Seeds

Upon addition of seeds or defatted meal of safflower seeds to whole milk a soft, creamy white, fresh cheese (24 g/100 mL of milk) with acceptable flavor was prepared. The coagulating agent was found to be an enzyme which was purified 61-fold as follows. The enzyme was extracted in 5 mM sodium phosphate buffer (pH 6) and precipitated in the presence of 50% saturated ammonium sulfate solution. The precipitates were dialyzed and applied to a column of Sephadex G-75. The defatted meal may be obtained inexpensively and it is already proven to be nontoxic.

The proteolytic enzyme renin from calf stomach and a renin substitute enzyme from fungus *Mucor miehei* are the major enzymes used in commercial cheesemaking (Kosikowski, 1977). Though several other renin-like enzymes from animals (Thomasow, 1971; Green, 1972), plant enzymes such as ficin, papain, and bromelin (Webb et al., 1974), and fungal enzymes (Iwasaki et al., 1968) have been examined for cheesemaking, all either failed to promote cheesemaking or resulted in undesirable cheese. Here we report the presence and partial purification of a milk-coagulating enzyme (renin-like) in safflower seeds.

EXPERIMENTAL SECTION

Cheesemaking. To 100 mL of pasteurized homogenized whole milk, 10 g of defatted meal of safflower seeds were added. The mixture was incubated at 50 °C for 10 min. The resultant cheese was drained in cheesecloth and salted. This experiment was repeated more than 20 times.

Enzyme Assay. To ensure a constant concentration of milk protein and minerals for assay, a solution of 10 dry defatted milk in 2% calcium chloride was used instead of milk for enzymatic assay during the course of enzyme purification. Enzymatic activity in the presence of dry defatted milk was similar to that of fresh milk.

Enzyme Sources. Any of three varieties of *Cartamus tinctorius* (safflower) seeds, Marand 3148, Mashhad 3150, and Arac 2811, were obtained from the Seed and Plant Improvement Institute, Karaj, Iran. The kernel of these varieties when added to the milk at 50 °C showed activity and produced cheese. Marand 3148 was found to be most active and thus this variety was used for enzyme purification.

Fractionation. The hulled ground seeds were used for oil extraction. Oil was extracted in a Soxhlet apparatus with hexane for 12 h. The excess of solvent was evaporated at 45 °C for 24 h. The defatted meal, which has the same activity as the seed, was extracted in ten parts of 5 mM sodium phosphate buffer (pH 6) and centrifuged at 1000 rpm for 60 min. The supernatant was brought to 50% saturation with ammonium sulfate, left overnight at 4 °C, and centrifuged again. The precipitates contained the activity, and the supernatant was inactive. The precipitates were dissolved in buffer (B in Table I) and dialyzed for 24 h against three changes of the same buffer.

The dialysate was centrifuged, and the supernatant was applied to a column (50 × 3.8 cm) of Sephadex G-75 and eluted at 42 mL/h with 5 mM sodium phosphate buffer (pH 6) (Figure 1). The fractions with highest activity were pooled and concentrated by mixing with dry Sephadex G-10, and the mixture was centrifuged.

RESULTS AND DISCUSSION

As shown in Table I, the enzyme was purified 61-fold. Addition of calcium chloride to the milk decreased the time required for cheese formation. Incubating the enzyme 24 h in the presence of EDTA diminished the activity. The maximum activity of the enzyme was found at pH 6, and it was active from pH 5 to 7. Further work characterizing the enzyme is in progress.

Table I. Enzymatic Activity During the Course of Purification[a]

extract	protein concn	sp act.
(A) first extract	45500	4.4
(B) $(NH_4)_2SO_4$ precipitate	2500	132.0
(C) dialysate	800	137.5
(D) Sephadex G-75	100	266.6

[a] Protein concentration was determined by measuring the absorbance of the solution and using the standard curve of albumin (Sigma) in concentration of 2-0.2%. The substrate was 5 mL of 10% dry defatted milk in 2% calcium chloride. Protein that coagulates 5 mL of milk in 1 min was taken as 400 units. Protein is in mg % and specific activity in enzyme unit/mg of protein.

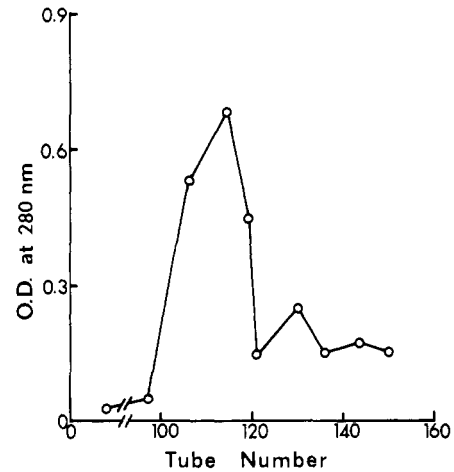

Figure 1. Elution pattern of enzyme from Sephadex G-75 column. Eighty milliliters of protein extract at a concentration of 8 mg/mL was applied to a column (50 × 3.8 cm) that was equilibrated with 5 mM phosphate buffer (pH 6). The flow rate was 42 mL/h, absorbance was at 280 nm, and 5-mL fractions were gathered.

The cheese produced (24 g of cheese/100 mL of milk) was edible and apparently appealing for consumption in that it was fresh, soft, white, and homogeneous and displayed an aromatic flavor pleasing to all six of the individuals who tasted it. The cheese dries at room temperature but could be refrigerated for 2–3 weeks. Although the whole seed could be used in the process, the resultant cheese had an undesirable oil flavor not found when defatted meal was used. Further work investigating the effect of heat ripening and other methods of improving the storage time and taste of the cheese is needed.

Significance. The search for new source of a renin substitute for commercial cheesemaking continues. So far substitutes from plants which are desirable have failed to

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produce edible cheese due to bitterness. Our finding of a renin-like activity in safflower seed is important because it makes sweet cheese and is present in the defatted meal of the seeds which are available in high quantities as a byproduct of oil production. Since the defatted meal is already in use for food fortification, this enzyme can be used in cheesemaking without being considered a potential threat to human health.

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Determination of the Herbicide Dinoseb in Fababeans

A analytical method for determining residues of the herbicide dinoseb (2-*sec*-butyl-4,6-dinitrophenol) in fababean (*Vicia faba* L.) green tissue and mature beans has been developed using electron-capture gas chromatographic detection. The fababeans were treated with preemergence applications of dinoseb amine at 4, 6, and 8 kg/ha. The maximum dinoseb residue found in the mature beans was 8 ppb, the limit of detection being 2 ppb based on a 1-g fresh-weight equivalent. Recoveries were in the order of 60% at the 50 ppb fortification level.

Fababeans (*Vicia faba* L.) are grown on a limited hectarage in Canada (less than 10 000 ha in 1977; Longmuir, 1978). Because of its high protein content, much of the fababean crop is harvested as silage (Winia, 1977). Dinoseb (2-*sec*-butyl-4,6-dinitrophenol)amine is presently registered in Canada for selective weed control in fababeans as a postemergence treatment. It has also shown potential for weed control in fababeans when used as a preemergence treatment, both in Prince Edward Island (Ivany and Sanderson, 1974; Ivany, 1975, 1976) and in Ontario (Anderson, 1975). However, residue data for this treatment are not available for registration purposes.

The present paper describes a highly sensitive method of analysis for the determination of dinoseb residues in fababeans. The method, based on that of McKellar (1970) for the determination of dinoseb residues in alfalfa, utilizes electron-capture gas chromatographic detection of dinoseb either as the free phenol or as the methyl ether. The method was used to determine dinoseb residues in both green tissue and mature beans after preemergence applications of dinoseb amine.

MATERIALS AND METHODS

Herbicide Treatments. Fababeans, variety Diana, were seeded on May 19, 1976, in 1.25 m × 6.0 m plots at the Agriculture Canada Research Station, Charlottetown, Prince Edward Island. Preemergence treatments of 4, 6, and 8 kg of dinoseb amine/ha were applied to the soil surface on May 26 using a hand-held small plot sprayer. The same treatments were similarly applied on May 27 to 12.6 m^2 plots located at the Nova Scotia Agricultural College, Truro, Nova Scotia, which had been seeded the previous day to fababeans (variety Acupearl). Each treatment at both locations was replicated four times and the herbicide was not incorporated into the soil.

Sampling. Green foliage samples were harvested at both locations on August 20 with additional sampling at Charlottetown on September 8. Ten plants, removed at random from each replicate, were chopped and thoroughly mixed, and the sample for residue analysis (approximately 0.25 kg) was taken. Mature beans were harvested on October 25 at Charlottetown. Composite samples (approximately 0.75 kg) for each treatment, made up of subsamples taken from the whole plot yield of each replicate, were taken for residue analysis. At Truro the mature beans, harvested on October 26, were picked at random from each replicate until the sample size (approximately 0.5 kg) was obtained.

Green foliage and mature bean samples from both locations were frozen in sealed polyethylene bags immediately after harvest, shipped to Regina in dry ice, and upon arrival, stored in a freezer at −10 °C until extraction.

Chemicals. All solvents were pesticide grade (Caledon Laboratories Ltd., Georgetown, Ontario, Canada). All steps involving benzene, which is very toxic to bone marrow, were carried out in or in front of a well-ventilated fume hood. The analytical grade dinoseb and dinoseb methyl ether were supplied by Dow Chemical of Canada, Ltd., Sarnia, Ontario, Canada.

The NaOH solution (pH 11) was prepared using a pH meter.

Sample Extraction. (a) *Beans.* Ten grams of mature fababeans (milled through a 1-mm screen), 50 mL of methanol, and 10 mL of 1.2 N H_2SO_4 were mechanically stirred in a 125-mL Erlenmeyer flask for 30 min at 70 °C (wax bath). The sample was cooled with stirring in an ice bath, filtered through a fritted glass Büchner funnel (porosity number 3), and washed twice with 20 mL of methanol, and the combined filtrates were taken to volume (100 mL) with methanol. Ten milliliters of the combined filtrates (equivalent to 1 g of plant tissue) and 10 mL of 2.5% NaCl solution were combined, extracted with 10 mL of benzene in a 60-mL separatory funnel, and centrifuged at 3000 rpm for 2 min, and the benzene layer was recovered

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