inhibitor from germinating barley seeds (Boisen et al., 1982) and for the thiol protease inhibitor of rat epidermis (Takeda et al., 1983).

The sites of interaction with trypsin and chymotrypsin even though are not mutually exclusive in the inhibitor, they appear to be partially distinct. Chemical modification studies reveal that amino groups are not essential for the action of the inhibitor on chymotrypsin whereas they are necessary for tryptic action, whereas guanido groups were essential for both the activities. Thus the chymotrypsin-inhibitor complex was found to inhibit trypsin, although to a reduced extent, whereas the reverse was not found to be true. Even though based on stoichiometric studies the inhibitor was expected to form a complex with trypsin in a 1:2 molar ratio, gel chromatographic studies did not provide evidence for such a stable complex. It is probable that at the second site of binding with trypsin the interaction is weak and could not be identified during the long period employed for gel chromatographic studies.

The chymotrypsin site of the inhibitor appears to be more exposed and easily susceptible to alterations. Thus heat treatment, exposure to high alkaline condition, treatment with pronase, and guanido groups modification resulted in more rapid loss of antichymotryptic activity. This is further supported by the observation that in the crude extracts of *Echinocloa* seeds there is rapid loss of antichymotryptic activity whereas the antitryptic activity was fully stable on storage. It has been reported earlier that the *Echinocloa* extracts did not possess antichymotryptic activity (Chandrasekher et al., 1982). The present observation on the disappearance of antichymotryptic activity explains this difference.

The relative ridigity of the trypsin site is supported by the observations on chemical modification. Heat treatment of the inhibitor prior to addition of amino group modifiers enhanced the rate of disappearance of the antitryptic activity. A similar observation has been made with alocasia trypsin/chymotrypsin inhibitor (Sumathi and Pattabiraman, 1977).

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We are grateful to Dr. A. Krishna Rao, Dean of this college, for his interest and encouragement.

Registry No. Trypsin inhibitor, 9035-81-8; proteinase inhibitor, 37205-61-1; chymotrypsin, 9004-07-3; trypsin, 9002-07-7.

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Isolation of an Amylase Inhibitor from *Setaria italica* Grains by Affinity Chromatography on Blue-Sepharose and Its Characterization

Ramanakoppa H. Nagaraj and Thillaisthanam N. Pattabiraman*

An α-amylase inhibitor from *Setaria italica* grains was purified 150-fold by chromatography on Blue-Sepharose after neutralization of the acid extract and ammonium sulfate fractionation. The inhibitor was found to be homogenous by polyacrylamide gel electrophoresis and gel chromatography on BioGel P-30. The molecular weight was found to be 24 K. SDS-PAGE showed that it is made up of two dissimilar polypeptides. Affinity chromatography on immobilized porcine pancreatic amylase and analysis showed that both the polypeptides are essential for the action of the inhibitor. The setaria inhibitor acted on human salivary amylase, human pancreatic amylase, and porcine pancreatic amylase but had no action on *B. subtilis* and *A. oryzae* amylases. It was labile to heat and to extreme acidic and alkaline conditions. Pronase, pepsin, trypsin, and α-chymotrypsin inactivated the inhibitor. Amino groups and guanido groups were found to be essential for its action.

Several of the proteinaceous α-amylase inhibitors from the plant kingdom have been purified by conventional methods (Buonocore et al., 1977; Warchalewski, 1983) or by affinity chromatography with immobilized α-amylase (Marshall and Lauda, 1975; Chandrasekher and Pattabiraman, 1983). During attempts to isolate the amylase inhibitor which was reported to be present in the millet

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Setaria italica (Chandrasekher et al., 1981) we observed that this inhibitor could bind selectively to Blue-Sepharose. In this communication we report this novel method for the isolation of an α-amylase inhibitor after preliminary purification by salt fractionation. The properties of the inhibitor are described.

MATERIALS AND METHODS

Setaria italica grains (variety Co-5) were procured from Tamil Nadu Agricultural University, Coimbatore, India. Porcine pancreatic amylase (twice crystallized), *Bacillus subtilis* α-amylase, *Aspergillus oryzae* α-amylase, pronase, sodium trinitrobenzenesulfonate (TNBS), ninhydrin, and Dalton Mark VI protein kit were purchased from Sigma Chemical Company, St. Louis, MO. Bovine trypsin (twice crystallized) and bovine α-chymotrypsin (three times crystallized) were from Worthington Biochemical Corporation, Freehold, NJ. Blue-Sepharose CL-6B was from Pharmacia Fine Chemicals, Uppsala, Sweden. *O*-Methylisourea (OMI) and 1,2-cyclohexanedione (CHD) were from Aldrich Chemicals, Milwaukee, WI. Affi-Gel 10 and Bio Gel P-30 were from Bio-Rad Laboratories, Richmond, CA. 5,5′-Dithiobis[2-nitrobenzoic acid] (DTNB) was purchased from Pierce Chemical Company, Rockford, IL. An acetone powder preparation of human pancreas was the source of human pancreatic amylase. Human salivary amylase was partially purified as described by Bernfeld (1955).

Amylase activity was measured by the method of Bernfeld (1955). One unit of amylase is defined as the amount of enzyme that will liberate 1 μmol of reducing equivalent (as maltose) under the assay conditions (pH 6.9, 37 °C, 5 min). For routine assay, 1.2 μg protein of human salivary amylase (HSA), 0.2 μg of porcine pancreatic amylase (PPA), 18 μg of human pancreatic amylase (HPA), 8.2 μg of *B. subtilis* amylase, and 15.4 μg of *A. oryzae* amylase provided four units of enzyme activity.

To estimate inhibitory activity, aliquots of inhibitor solution were preincubated with the target enzyme in a volume of 1.5 mL in the presence of 30 μmol of phosphate buffer, pH 6.9, and 10.5 μmol of NaCl for 20 min at 37 °C. Controls were run simultaneously. The decrease in enzyme activity was a measure of inhibitory activity. One unit of inhibitor is defined as the amount that decreased amylase activity by one unit.

Protein was estimated by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Isolation of the Inhibitor. All operations were carried out at 4 °C unless stated otherwise. *Setaria* seed powder (400 g) was extracted with 1600 mL of 0.1 N HCl containing 0.15 M NaCl. The homogenate was stirred mechanically for 2 h and centrifuged for 30 min at 10 000*g*. The supernatant (acid extract) was collected and the pH was adjusted to 7.0 with 5 N NaOH. Protein precipitated was removed by centrigation at 10 000*g* for 10 min. To the clear supernatant (neutral extract) was added solid ammonium sulfate (329 g) to 50% saturation. The solution was allowed to stand for 24 h and centrifuged at 10 000*g* for 30 min. The precipitate was dissolved in 0.05 M Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl and dialyzed against the same buffer for 24 h. The dialyzed solution (ammonium sulfate fraction) after centrifugation at 10 000*g* was passed through a column of Blue-Sepharose (0.9 × 24 cm^2, bed volume 15 mL, flow rate 30 mL/h) equilibrated with 0.05 M Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl. The column was washed with 200 mL of the equilibration buffer. The inhibitor was eluted with the buffer containing 1.5 M NaCl. Fractions of 15 mL were collected and assayed for protein and amylase inhibitory activity. The elution profile is shown in Figure 1. The 20th fraction which had the inhibitory activity was concentrated by ultrafiltration to 4.0 mL, dialyzed against 0.05 M Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl, and used for further studies.

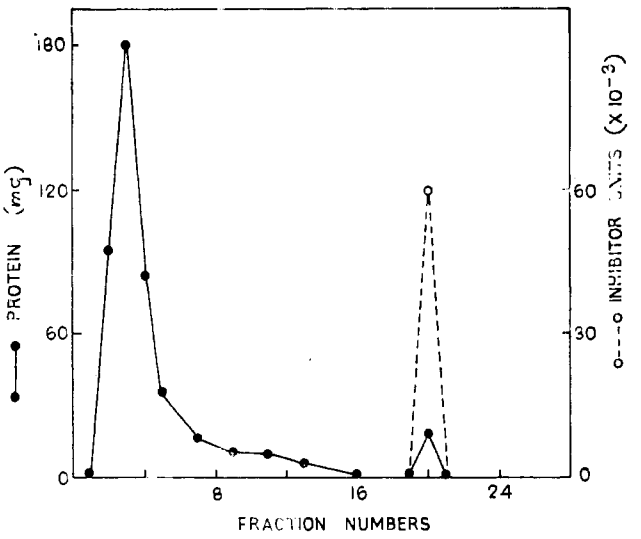

Figure 1. Affinity chromatography of ammonium sulfate fraction on Blue-Sepharose CL-6B. Experimental details are described under Materials and Methods.

Electrophoresis. The purified inhibitor was subjected to polyacrylamide gel electrophoresis (PAGE) in Tris-glycine buffer, pH 8.6, with 5% gel for 6 h at a current of 5 mA per tube. Sodium dodecyl sulfate (SDS)-PAGE with and without mercaptoethanol (1%) was done for 8 h at a current of 7 mA per tube with 10% gel in 0.1 M sodium phosphate buffer, pH 7.2. The purified inhibitor was also subjected to cellulose acetate electrophoresis at pH 8.6 (0.05 M barbitone buffer) for 1.5 h at a current of 1.5 mA per cm of the strip. The protein bands were stained with Coomassie Brilliant Blue R-250 (0.25% in methanol–acetic acid–water, 5:1:5). Destaining was done by washing with 7% acetic acid.

Molecular Weight Determination. This was done by SDS-PAGE with Dalton Mark VI (containing lysozyme, lactoglobulin, trypsinogen, pepsin, ovalbumin, and bovine serum albumin) for standards. Molecular weight was also assessed by gel chromatography on Bio-Gel P-30 (0.9 × 60 cm^2 column, bed vol 38 mL) with 0.05 M Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl as eluant. Cytochrome c, myoglobin, α-chymotrypsin, and pepsin were used as standards. Fractions (1 mL) were collected at a flow rate of 6 mL/h.

Stability of the Inhibitor. The inhibitor (2 μg of protein) in a volume of 0.2 mL was exposed to various temperatures for 10 min. After cooling the samples, residual inhibitory activity was determined. The inhibitor (2 μg protein) was incubated in the presence of 10 μmol each of HCl (pH 1.0), HCl–KCl buffer (pH 2.0), citrate buffer (pH 3.0 and 5.8), acetate buffer (pH 4.0 and 5.0), phosphate buffer (pH 6.9), Tris-HCl (pH 8.0, 9.0, and 10.0) and NaOH (pH 12.0) in 0.2 mL at 4 °C for 24 h. Phosphate buffer (200 μmol) was added to the systems and amylase inhibitory activity was then estimated.

Effect of pH on the Interaction of Amylase and Inhibitor. The inhibitor (4 μg of protein) and HSA (1.2 μg of protein) were preincubated for 20 min in 0.2 mL of different buffer systems as described above at 37 °C. The enzyme action was initiated by the addition of 1.7 mL of 0.3% starch solution containing 80 μmol of phosphate buffer, pH 6.9, containing 10 μmol of NaCl. Controls

Table I. Purification of *Setaria* Amylase Inhibitor[a]

stage	volume, mL	protein, mg	inhibitor units ($\times 10^{-3}$)	specific activity	yield, %
acid extract	1230	7010	148.0	21.0	100
neutral extract	1050	3570	105.0	29.5	71.4
$(NH_4)_2SO_4$ fraction	42	604	77.7	128.0	52.3
Blue-Sepharose fraction	15	19.2	60.0	3120.0	40.0

[a] The inhibitor units are based on action against HSA.

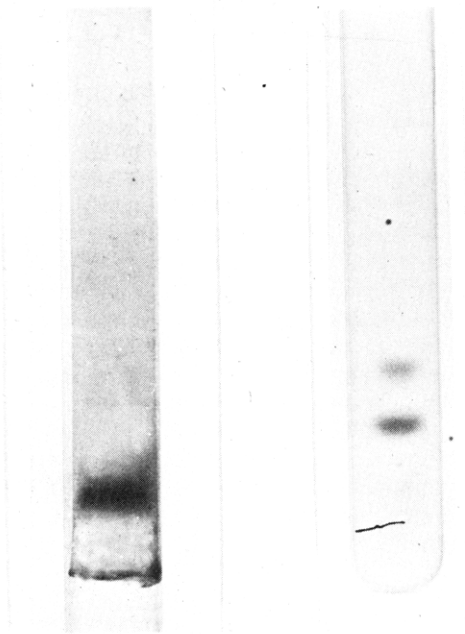

Figure 2. (A) Polyacrylamide gel electrophoresis of the inhibitor. (B) SDS-Polyacrylamide gel electrophoresis of the inhibitor with mercaptoethanol. Experimental details are described under Materials and Methods.

without inhibitor were run simultaneously.

Effect of Proteolytic Enzymes. The inhibitor protein (20 μg) was treated with bovine trypsin (10 μg), bovine α-chymotrypsin (10 μg), or pronase (20 μg) at 37 °C at pH 7.6. At varying time intervals, aliquots were withdrawn and assayed for residual inhibitory activity. Similarly, inhibitor was treated with pepsin (20 μg of protein) at pH 2.0. In all cases, inhibitor controls and enzyme controls were run simultaneously.

Chemical Modification Studies. Lysine residues were modified by treatment with TNBS (Haynes et al., 1967) and OMI (Kimmel, 1976). Arginyl residues were modified with CHD (Abe et al., 1978) and ninhydrin (Chaplin, 1976). Sulfhydryl groups were modified with DTNB (Ellman, 1959). After dialysis the modified inhibitor was assayed for inhibitory activity against HSA. Controls without modifiers or without inhibitor were run simultaneously.

RESULTS

The setaria amylase inhibitor was purified nearly 150-fold with a recovery of 40% of the activity. Data on the purification process are shown in Table I. The chromatographic step on Blue-Sepharose accounted for a rapid and facile purification with a 25-fold increase in specific activity.

The inhibitor on PAGE at 8.6 (Figure 2 part A) or cellulose acetate electrophoresis at pH 8.6 (data not shown) moved as a single protein band. During gel chromatography on BioGel P-30, the inhibitor was eluted as a sharp

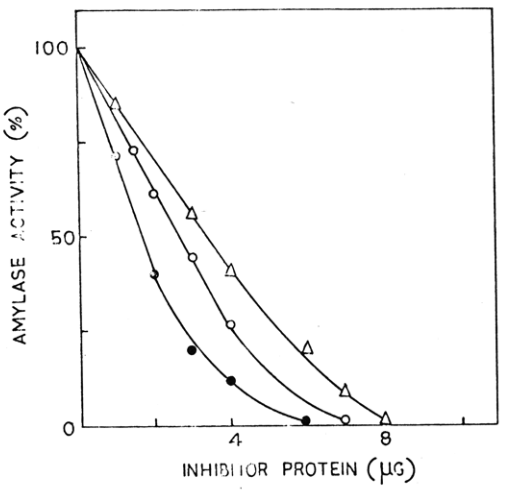

Figure 3. Effect of varying concentrations of inhibitor on the activity of HSA (●–●), HPA (△–△), and PPA (○–○). Experimental details are given under Materials and Methods.

single peak with a constant ratio of protein and inhibitory activity across the peak (data not shown). These data attest to the homogeneity of the inhibitor. The molecular weight of the inhibitor was determined to be 24 K by gel chromatography.

On SDS-PAGE both in the presence and in the absence of β-mercaptoethanol the inhibitor dissociated into two protein bands (Figure 2 part B). The molecular weights of the two fractions were found to be 12 K and 16 K. Attempts to elute the bands and locate the inhibitory activity in any one of the protein fragments were not successful. To test the possibility whether one of the fragments is a contaminant protein associated with the inhibitor under native conditions, the following experiment was performed. Porcine pancreatic α-amylase (3 mg) was immobilized on Affi-Gel 10 (wet volume 2.5 mL), packed into a column and equilibrated with 0.02 M sodium phosphate buffer, pH 6.9. The purified *Setaria* inhibitor (1.0 mg) in 1.5 mL of the same buffer was passed through the column. The washings did not contain any protein or inhibitory activity. The bound inhibitor was eluted with 25 mL of phosphate buffer containing 1 M maltose. The eluant was extensively dialyzed against 0.02 M phosphate buffer, pH 6.9, and concentrated by ultrafiltration. The concentrate which had amylase inhibitory activity was subjected to PAGE and SDS-PAGE. PAGE showed a single protein band as in the case of the native inhibitor. During SDS-PAGE the inhibitor resolved as before into two protein bands. These data indicate that the two polypeptides that constitute the inhibitor are integral parts, essential for the biological activity.

The purified inhibitor acted on HSA, HPA, and PPA. The relative ratios of inhibition were 1.0:0.5:0.7 based on data in the linear range of action. The magnitude of inhibition was linear with respect to inhibitor concentration up to 70%. Beyond this point the inhibition was progressive but nonlinear, reaching nearly 100% inhibition at high concentrations (Figure 3). The purified inhibitor had no effect on *B. subtilis* and *A. oryzae* amylases at concentrations up to 10 μg of protein. The inhibitory activity was dependent on the time of preincubation of the inhibitor with the susceptible enzymes. A minimum of 20 min preincubation was necessary to elicit optimal response. For example, with HSA the percent inhibition without preincubation was 16% of the inhibition after 20 min preincubation. Pretreatment of the inhibitor with starch solution for 10 min before the assay resulted in complete

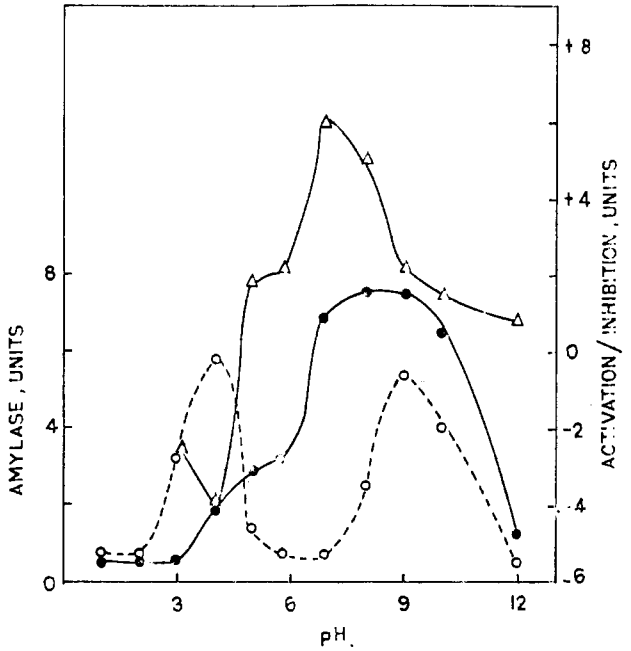

Figure 4. Effect of pH on the preincubation of inhibitor with HSA. ●–● indicates the activity of HSA, Δ–Δ indicates the activation/inhibition of HSA in presence of inhibitor, and O–O indicates the activity of HSA in the presence of inhibitor. Experimental details are given under Materials and Methods.

disappearance of the inhibitory activity.

The inhibitor was not very heat stable. Heat treatment at 50 °C for 10 min and 70 °C for 10 min resulted in the loss of 50% and 100% of the inhibitory activity, respectively. On storage at 4 °C for 2 weeks there was 50% loss in activity. The inhibitor was maximally stable around pH 7.0. On either side there was sharp fall in activity. The percent residual activity on exposure to pH 4.0, pH 9.0, and pH 10.0 (24 h, 4 °C) were 16%, 16%, and 0%, respectively. Interestingly below pH 4.0, the inhibitor was relatively more stable. On exposure to pH 1.0 and 2.0 the relative losses of activities were 50% and 60%. It is probable the inhibitor is precipitated at pH 4.0 resulting in apparently more loss of activity.

The effect of pH on the interaction of the inhibitor and HSA (preincubation system) is represented in Figure 4. Optimal pH for interaction was around pH 6.9. In the alkaline range the inhibition declined progressively. In the acidic range the picture was complicated by the instability of the enzyme itself. Interestingly, the amylase activity in the test systems (+ inhibitor) was more at pH 3.0 and 4.0, indicating that the inhibitor protects the enzyme from inactivation.

The inhibitor was found to be easily inactivated by pronase, pepsin, trypsin, and α-chymotrypsin (Figure 5). The data on the effect of chemical modification on the activity of *Setaria* inhibitor are shown in Table II. TNBS caused a rapid loss of activity indicating that amino groups of the inhibitor are essential for its function. However, OMI did not cause any loss of the inhibitory activity. This can be due to the following reasons. While reaction with TNBS causes the substitution of a bulky aromatic groups, OMI converts lysine residue to a homoarginine residue causing relatively small addition to the size of the side chain and at the same time retaining the positive charge. Ninhydrin under conditions that specifically modify the guanido group (Chaplin, 1976) also caused a rapid loss of inhibitory activity, implicating guanido groups in the interaction of the inhibitor with amylase. However, CHD, another guanido group modification, did not abolish the

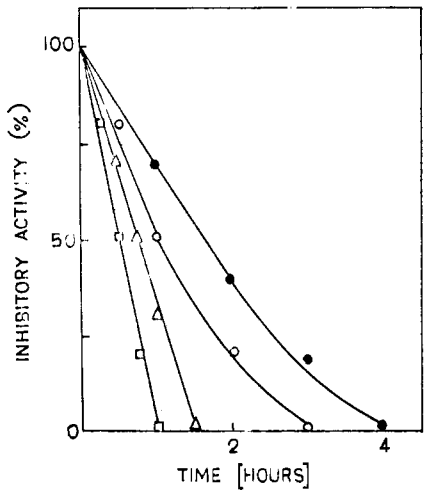

Figure 5. Effect of various proteolytic enzymes on the inhibitor: (●–●) trypsin; (O–O) chymotrypsin; (□–□) pronase; (Δ–Δ) pepsin. Experimental details are given under Materials and Methods.

Table II. Effect of Chemical Modifiers on the Inhibitor

modifier	time of treatment, h	percent residual inhibitory activity against HSA
TNBS	0.5	46
TNBS	1.0	23
TNBS	2.0	0
OMI	2.0	100
OMI	6.0	100
ninhydrin	0.5	56
ninhydrin	1.0	22
ninhydrin	2.0	0
CHD	2.0	100
CHD	7.0	100
DTNB	4.0	100
DTNB	8.0	100
mercaptoethanol	3.0	100
mercaptoethanol	6.0	100

[a] The details are described under Materials and Methods.

activity on treatment up to 7 h. Longer treatments could not be tested since the inhibitor per se was unstable at pH 9, the condition used for modification. It has been observed that ninhydrin is far more reactive in inactivating the proteinase inhibitor (Udupa and Pattabiraman, 1984) and amylase inhibitor (Kutty and Pattabiraman, 1984) from *Echinocloa* seeds. DTNB and β-mercaptoethanol treatments did not have any effect on the inhibitor, ruling out the roles of sulfhydryl groups and disulfide bridges for the functional activity of the inhibitor.

The mode of inhibition of HSA by the *Setaria* inhibitor was found to be noncompetitive. The K_i value was calculated to be 0.24×10^{-8} M based on a molecular weight of 24 K.

DISCUSSION

Even though several proteins and enzymes are known to bind specifically and reversibly to Blue-Sepharose (Travis and Pannell, 1973; Easterday and Easterday, 1974), this is the first report of an α-amylase inhibitor exhibiting such a property. It will be of interest to examine whether the amylase inhibitors as a group will display an affinity to Blue-Sepharose.

The *Setaria* amylase inhibitor in many respects resembles the other plant amylase inhibitors in its physicochemical properties. Like the "0.19" inhibitor from wheat (Petrucci et al., 1978) it is made up of two subunits which are not identical. The subunits in this case also are associated noncovalently. In this respect it differs from the

rye inhibitor (Granum, 1978) and pearl millet inhibitor (Chandrasekher and Pattabiraman, 1983) which are known to be made up of two subunits but linked by disulfide bridges.

Like the pearl millet inhibitor (Chandrasekher and Pattabiraman, 1983) the *Setaria* inhibitor was found to be relatively heat labile and susceptible to proteolytic digestion. In view of this, the inhibitor may not be of much nutritional significance. An interesting property indicated here is the capability of the inhibitor to protect the target amylase from inactivation under acid conditions.

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The keen interest and encouragement of Dr. A. Krishna Rao, Dean of this college, are acknowledged.

Registry No. α-Amylase, 9000-90-2; Blue-Sepharose, 66456-82-4.

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Composition of Leaf Oils in the Genus *Parthenium L.* Compositae

Junji Kumamoto, Rainer W. Scora,* and Wilhelmus A. Clerx

Essential leaf oils were steam distilled from seven Parthenium species: *P. tomentosum*, *P. fruticosum* var. *trilobatum*, *P. schottii*, *P. incanum*, *P. argentatum*, *P. confertum* var. *lyratum*, *P. hysterophorus*. Only *P. argentatum* and *P. confertum* contain bornyl acetate, thus providing perhaps a link between the woody section Parthenichaeta and the herbaceous section Argyrochaeta.

While the development of synthetic rubber alleviated our country's total dependence on foreign sources of natural rubber, it increased our dependence on petrochemicals which are in a state of irreversible depletion. There are some applications where only natural rubber can be used, and this keeps the U. S. dependent on foreign sources for this critical raw material. Because the prices of both petroleum and natural rubber are subject to influences beyond our control, any developments which will partially free us from this dependence will be of benefit to our national interests. The 1983 Critical Agricultural Materials Act (H. R. 2733) provided an impetus for continuing the development of a domestic source of natural rubber. It will have the advantage of being a renewable resource and diminish our demand on the depleting fossil petrochemicals (Calvin, 1976).

The identification of any useful byproducts will enhance the chances for the successful commercialization of the production of natural rubber from guayule, *Parthenium argentatum* A. Gray.

Present breeding programs are directed at selecting guayule lines for increased rubber content, producing hybrids with increased biomass, and also hybrids with a greater cold tolerance to extend the range of cultivations in the US.

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In guayule, the rubber is found in the stems, so the leaves are initially stripped off and discarded. Volatile oils can be distilled from this waste. The components in the oils must first be identified before any assessment of their value can be made. The possibility of increasing desirable oil constituents by breeding should not be overlooked. Interest in the volatile oils from *P. argentatum* dates back to Alexander (1911), Haagen-Smit and Siu (1944), and Scora and Kumamoto (1979). Dominguez et al. (1971) identified limonene and α-pinene from the steam distillate of fresh material from *P. incanum* H.B.K. We have recently reported on the identification of bornyl acetate from *P. argentatum* and *P. confertum* var. *lyratum* A. Gray (Kumamoto and Scora, 1984).

We wish to report on our results of a survey of leaf oils from seven species in the genus *Parthenium*. The taxa examined here are *P. tomentosum* DC, *P. fruticosum* Rollins, *P. schottii* Greenm., *P. incanum* H.B.K., *P. argentatum* A. Gray, *P. confertum* var. *lyratum* A. Gray, and *P. hysterophorus* L.

EXPERIMENTAL PROCEDURE

Plants of seven species were grown in a University of California, Riverside, lathhouse. Samples (500 g) of healthy, mature leaves were harvested between 0830 and 1000 h and immediately ground and steam distilled under an atmosphere of carbon dioxide, and the oils collected in a Clevenger trap. A Varian 1520 GC with a thermal conductivity detector and a linear temperature program of 1

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