CRYSTALLIZATION OF FRACTION I PROTEIN FROM TOBACCO BY A SIMPLIFIED PROCEDURE

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1. Introduction

Tobacco leaves are relatively high in protein [1]. The major protein of leaf tissue is Fraction I protein. Recently a theoretical model for improving leaf quality and usability was proposed in which the desirability of removal of certain components from tobacco leaves was suggested [2]. Among these removable components is protein, including Fraction I protein. The new homogenized-leaf curing process [3] provides the possibility for extraction of many presumed undesirable compounds including Fraction I protein. This suggests a convenient large-scale source of Fraction I protein for use as a food source [4] and for possible medical and industrial uses.

Although a simple procedure has been reported for crystallization of Fraction I protein from tobacco [5], it is not adaptable to large-scale production because of the necessity to concentrate the protein by time-consuming ultrafiltration and for salt removal by dialysis. The procedure reported here is further simplified by eliminating these steps, and possibly may be used in combination with the homogenized-leaf curing process to obtain an improved tobacco product and to yield crystalline Fraction I protein as a valuable by-product.

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2. Materials

Burley tobacco (*Nicotiana tabacum* L., cv. Ky 14) was grown in a soil bed in the greenhouse. A highly fertile soil with supplemental nitrogen added was used to obtain plants high in protein. Leaves were harvested from bud to full-flower stage. Only dark green, fully-expanded or nearly fully-expanded leaves were used.

A column containing Sephadex G-50 coarse, equilibrated with 25 mM Tris–HCl buffer, pH 7.4, containing 0.2 mM EDTA, was used for desalting. The column bed-volume was approximately 3 times the volume of the extract to be desalted. A flow rate of no more than 1 ml/min/cm^2 of column cross-section was used. The CO_2 content in the buffer used for equilibration of the column and for elution of the protein was diminished by evacuation to approximately 5 cm Hg pressure. Between each run the column was washed with 0.1 N KOH and then with the above-described buffer, until the column effluent was pH 7.4.

3. Results

Figure 1 illustrates the general simplified procedure for crystallization of Fraction I protein in high yield from fresh tobacco leaves. The crude protein extract was prepared by grinding 1600 g leaf lamina (midrib removed) in a commercial size Waring Blendor containing 160 ml 5 M NaCl. With the blender at low speed, leaves were added gradually as rolls, along with sodium metabisulfite (3.2 g) and enough 0.5 M Tris to maintain a pH of approximately 6.0. A total of

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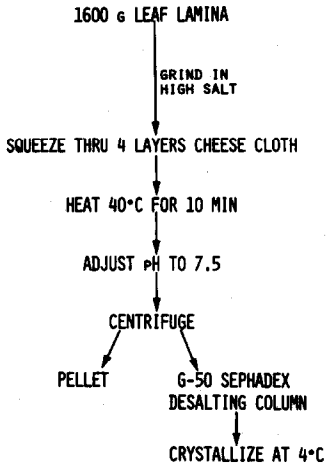

Fig.1. Scheme for crystallization of Fraction I protein from tobacco leaves.

about 30 ml Tris was needed, depending on the acidity of the leaves. After all leaves were added and a slurry obtained, the crude slurry was blended at high speed for 30 s. The resulting slurry was squeezed through 4 layers of cheese cloth in 300–400 ml batches to remove the pulp. The green filtrate (about 1300 ml) was then heated rapidly with stirring to 40°C and maintained at this temperature for 10 min. The suspension was then cooled in an ice bath to approximately 20°C, 3.2 g of EDTA added, and the pH adjusted to 7.5 with 0.5 M Tris (about 70 ml). The suspension was centrifuged for 20 min at 45 000 X *g* in a Type 21 rotor in a Model L Spinco centrifuge (longer centrifugation times can be used in slower speed centrifuges). The supernatant was decanted, care being taken to avoid decanting any of the green pellet.

The extract was then desalted by passing through a Sephadex G-50 column as described in the Materials section. Fractions were collected when protein first appeared in the effluent and collection continued until proteins were absent from the effluent. The last half of the extract from the column was caught in 2–3 separate fractions. By doing so, only the last part of the batch will fail to crystallize if a poor flow-pattern is obtained. Sufficient 1 M $NaHCO_3$ and 1 $MgCl_2$ were added to the fractions to yield 5 mM and 0.5 mM, respectively. After 1–3 days at approximately 4°C, crystals had formed and settled (fig.2). The liquid was then carefully decanted and the crystals were washed with, pH 7.4, Tris–HCl buffer (0.025 M) containing 0.2 mM EDTA. The washed crystals can be collected by gentle centrifugation or

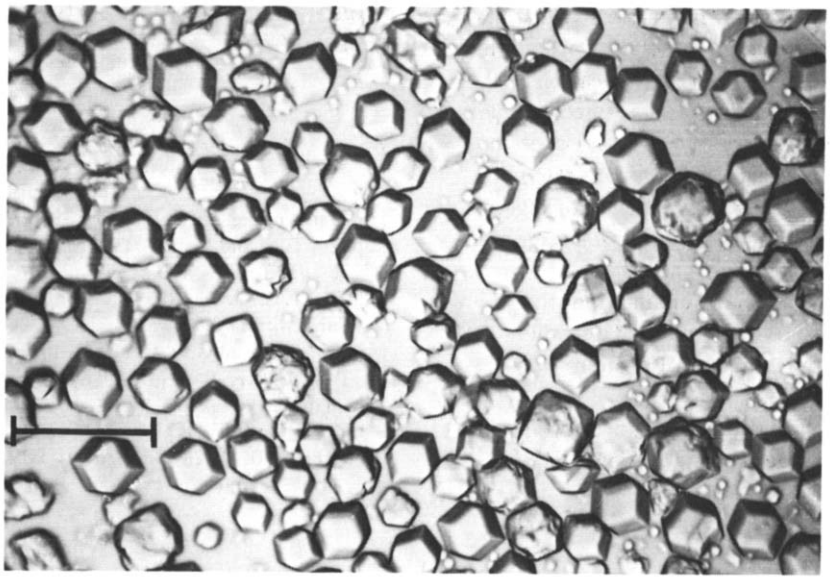

Fig.2. Unwashed crystals of Fraction I protein from the effluent of the Sephadex column (bar = 0.1 mm).

by allowing them to settle for several hours. Yields of up to 6 mg/g fresh weight of leaves have been obtained and the ribulose-1,5-bisphosphate carboxylase activity is similar to that from crystals obtained by the earlier procedure.

4. Discussion

In the original procedure described for crystallization of Fraction I protein [5], gel filtration was employed to decrease salt concentration from approx. 0.5–0.2 M NaCl and to remove phenolics from the protein extract [6]. Crystallization of Fraction I protein from buffered 0.2 M NaCl required concentration of the protein by ultrafiltration and subsequent dialysis to further decrease the salt concentration.

In this procedure, gel filtration is employed simultaneously to desalt completely the protein extract and to remove phenolics and possibly other low molecular weight substances that possibly prohibit crystallization of Fraction I. This leads directly to Fraction I crystallization in high yield, thus eliminating requirements for time-consuming ultrafiltration and dialysis.

Recently, renewed interest in Fraction I has centered on: (1) its dualistic function as a carboxylase and oxygenase [6] and (2) the possibility of large-scale production of Fraction I protein as a valuable by-product from the new homogenized-leaf curing process [3]. It has been suggested that pure crystalline Fraction I protein may have nutritional as well as medical applications [4].

The described procedure has not yet been shown to be applicable for recovery of Fraction I protein from the homogenized-leaf curing process. However, the simplicity of the method described here suggests that it may be applicable for the proposed large-scale production of this protein.

Independent of commercial application, the procedure may be useful for providing a readily available source of pure Fraction I protein for biochemical investigations.

It should be noted that the procedure described here employs G-50 rather than G-25 Sephadex [5]. For a given column dimension, G-50 was much more effective than G-25 for the direct crystallization of Fraction I from the column effluent. Since larger columns of G-25 were as effective as smaller columns of G-50, the results suggest that the extract contains a compound(s) (not NaCl) which must be removed for crystallization to occur. Apparently, G-50 is more effective than G-25 for gel exclusion of this suggested unknown compound(s).

Though this procedure is applicable to burley tobacco, the procedure, with slight modifications, has been used successfully with Maryland-type tobacco by Dr S. D. Kung, University of Maryland Baltimore County and with Turkish-type tobacco by Dr S. G. Wildman, University of California, Los Angeles (personal communications). Preliminary experiments with spinach in this laboratory have failed to yield crystalline Fraction I.

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