J. Agric. Food Chem. 1982, 30, 770–771

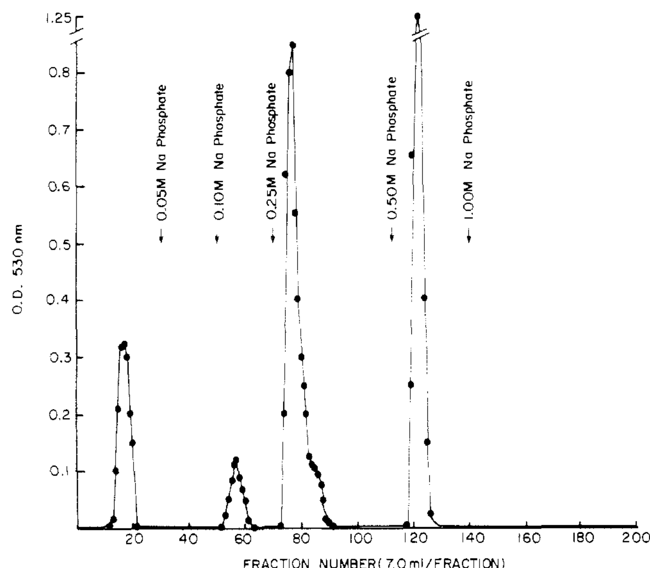

Figure 1. DE-52 ion-exchange chromatography of pectin. For details, see the text.

galacturonic acid content of these fractions. However, these fractions differ from each other in their neutral sugar composition. Of interest is the neutral sugar composition of fraction I (eluted with 0.025 M sodium phosphate) and fraction IV (eluted with 0.5 M sodium phosphate). These fractions were found to be rich in their rhamnose and poor in their galactose content. The sugar composition of unfractionated pectin was found to be similar to the sugar composition reported earlier for citrus pectin (Aspinall et al., 1968). It is important to point out at this juncture that the chemistry of the neutral sugar components of the commercially available pectin described here is remarkably different from the chemistry of pectin isolated by employing gentle extraction procedures (Baig et al., 1980). As noted earlier by several investigators (Aspinall, 1970), this difference appears to be caused by differences in the methodology used to extract pectin from grapefruit.

The results obtained from these studies clearly affirm the heterogeneous nature of commerically available citrus pectin and conclusively indicate that the grapefruit pectin is composed of a number of pectic polysaccharides differing in their methoxy as well as neutral sugar composition. In view of this observed heterogeneity of dietary citrus pectin, it is apparent that investigative studies aimed at evaluating the dietary role of pectin in lowering cholesterol levels in man and laboratory animals must take into consideration the fact that pectin is not a single identity but a mixture of a number of pectic polysaccharides. In addition, since pectin represents the material found in the primary cell wall of plants, it is highly possible that the qualitative nature as well as quantity of various pectic polysaccharides found in pectin may vary with the degree of maturity/differentiation of the plant source from which dietary pectin is extracted. Meaningful evaluation of the dietary role of pectin could therefore be only achieved by using individual pectic polysaccharides of known physicochemical characteristics.

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Received for review July 16, 1981. Accepted March 26, 1982. The Florida Citrus Commission provided funds for this research.

Alfalfa Trypsin Inhibitor Inhibits Alfalfa Leaf Proteinase

The alfalfa (*Medicago sativa*) leaf trypsin inhibitor inhibits the alfalfa leaf proteinase. This inhibition is strictly dependent on ionic strength.

In a previous paper we brought up evidence suggesting that the alfalfa leaf neutral proteinase is a serine trypsin-like proteinase (Tozzi et al., 1981). This observation prompted us to investigate the inhibitory effect of the alfalfa leaf trypsin inhibitor described by Chang et al. (1978) on the endogenous proteinase. In the present communication we report that alfalfa trypsin inhibitor also inhibits alfalfa endogenous proteinase.

EXPERIMENTAL SECTION

Materials. The alfalfa trypsin inhibitor was purified according to Chang et al. (1978). The alfalfa proteinase was purified according to Tozzi et al. (1981). Trypsin, N^{α}-benzoyl-DL-arginine-*p*-nitroanilide (DL-BAPNA), and

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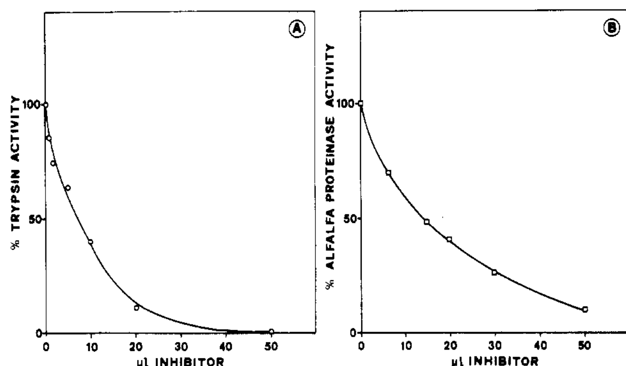

Figure 1. Percent residual proteolytic activity as a function of increasing concentrations of inhibitor. The assays were carried out at 37 °C by using 3×10^{-9} M active site concentration of trypsin (A) or alfalfa proteinase (B) assayed according to Chase and Shaw (1970) and the indicated amounts of inhibitor solution. The reaction was started by addition of the substrate.

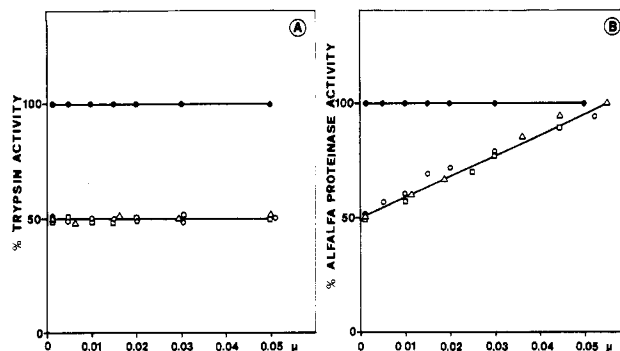

Figure 2. Dependence of trypsin (A) or alfalfa proteinase (B) inhibition on ionic strength. Assays were carried out as in Figure 1 in the absence (●) or in the presence of a constant volume of inhibitor solution (50% inhibition) and increasing concentrations of NaCl (○), Na_2SO_4 (△), and NH_4Cl (□) solutions at the reported value of μ.

p-nitrophenyl *p*′-guanidinobenzoate hydrochloride (*p*-NPGB) were purchased from Sigma Chemical Co. (St. Louis, MO). The active-site concentration of both trypsin and alfalfa proteinase were measured according to the method of Chase and Shaw (1970) using *p*-NPGB as the active-site serine titrator.

Enzyme Assay. The spectrophotometric assay was carried out at 37 °C according to Erlanger et al. (1961). The assay mixture contained 0.001 M Tris-HCl, pH 8, 3×10^{-9} M active-site concentration of proteinase or trypsin, and the amounts of inhibitor solution and/or salt concentrations indicated in the legend to the figures. The reaction was started by addition of DL-BAPNA solution to a final concentration of 2.2 mM. The final volume was 1 mL. The increase of the optical density at 405 nm was recorded. The proteolytic activity is expressed as micromoles of substrate transformed per minute per milliliter at 37 °C. The inhibitor activity is expressed as percent residual proteolytic activity.

RESULTS AND DISCUSSION

In Figure 1 the inhibitory effect of alfalfa leaf inhibitor on both trypsin and endogenous proteinase is reported. Upon addition of increasing amounts of inhibitor solution, the tryptic activity falls more rapidly than the endogenous alfalfa proteinase activity, and it is completely abolished by addition of 50 µL of inhibitor solution. The 50% inhibition is obtained by addition of 7.5 and 14 µL of inhibitor solution for the trypsin and the alfalfa proteinase, respectively. This differences may be explained on the basis of the effect of ionic strength. Figure 2 shows the effect of ionic strength on the inhibition exerted by the alfalfa inhibitor on both trypsin and endogenous proteinase. Neither of the two activities is affected by salt concentration. Similarly, the inhibitory effect on trypsin is independent of ionic strength at least up to a value of $\mu = 0.05$. When the alfalfa endogenous proteinase is assayed in the presence of the inhibitor (50% inhibition) with increasing salt concentrations, the inhibitory effect linearly decreases, and it is completely abolished at an ionic strength of 0.05. No effect in the removal of the inhibitory effect was shown by the nature of the ions.

The physiological role of the proteolytic enzymes and of their control by endogenous proteic inhibitors has been widely studied in both animal and bacterial cells (Desnuelle, 1960). Also, the inhibitory effect of plant proteic inhibitors on the serine endopeptidase of both mammalian origin and bacterial origin has been studied (Stevens et al., 1974; Wilson and Laskowski, 1974), but no regulation of proteolytic enzymes by endogenous proteic inhibitors has so far been described in plant leaves (Ryan, 1980). Two main hypotheses on the significance of the presence of protease inhibitors in the plant kingdom have been postulated. The attention of nutritionists has been focused on the possible role that the inhibitors might play in determining the nutritive value of plant proteins (Liener, 1978; Kakade et al., 1973). Furthermore, the findings of Ryan and co-workers that the synthesis of inhibitors is hormone regulated in plant leaves and that such regulation is triggered by wounding allowed the hypothesis that proteolytic inhibitors are involved in defense mechanisms (Ryan, 1980). The reported inhibitory effect in vitro at low ionic strength suggests that the alfalfa leaves proteinase could possibly be regulated in vivo. Further studies are in progress to establish the molecular mechanisms of the observed inhibitory effect. Preliminary observations suggest that the ionic strength affects the secondary structure of the inhibitor molecule.

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Received for review September 17, 1981. Accepted February 24, 1982. This work was supported by grants from the Italian C.N.R. (Progetti Finalizzati).