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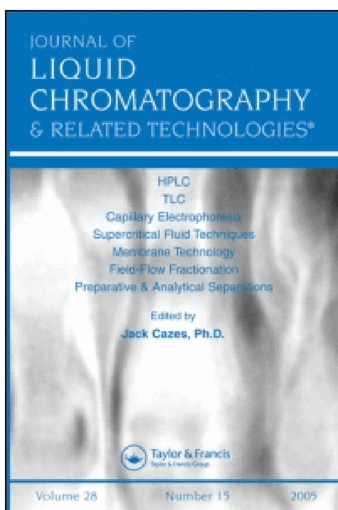

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QUANTITATIVE DETERMINATION OF THE α-AMYLASE INHIBITOR FROM *PHASEOLUS VULGARIS* USING SIZE EXCLUSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A quantitative determination of the α-amylase inhibitor from kidney beans, a glycoprotein (MW 49,000), by the use of a TSK-SW 3000 column is described. A correlation coefficient of 0.986 was obtained when the HPLC method was compared with a biological assay using Beckman Enzymatic Amylase-DS Reagent Kit. The within-day and between-day coefficients of variation were 4.8-8.9% and 3.7-11.6%, respectively. The results indicate this method is a reliable assay that has advantages over the biological assays presently available.

INTRODUCTION

High performance size exclusion chromatography has been used quite successfully in the separation of proteins (1-4). There have been, however, a limited number of reports in which proteins have been quantitatively assayed by HPLC (5,6). Size exclusion chromatography was chosen for the development of our quantitative chromatographic assay of the α-amylase inhibitor from kidney beans (*Phaseolus vulgaris*) because of the reportedly high degree of recovery of proteins in their active form (2,4) and the need to measure the biological activity of the inhibitor.

There have been numerous α-amylase inhibitors from plant and animal sources identified (7-10). This group of substances gained

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notoriety when they were introduced into the market as 'starch blockers' to be used as dietary aids in weight control, but they were subsequently removed from the market by the FDA. The efficacy of these products as starch blockers is controversial (11-14). Other clinical and agricultural applications of α-amylase inhibitors have also been investigated (15-17).

The α-amylase inhibitor from kidney beans is a glycoprotein with a molecular weight of 49,000 and characteristics of its inhibitory properties on α-amylase have been reported (7).

MATERIALS AND METHODS

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The HPLC system was composed of a model 825 pump and model 226 UV (280 nm) detector (Instrument Specialties Company); model 210 injector and Spherogel TSK-SW 3000 size exclusion column, 300 x 7.5 mm (Beckman Instruments); and model 3390A integrator (Hewlett-Packard Company). The mobile phase flow rate was 1 ml/min and consisted of 85% of 0.15 M sodium chloride in 0.01 M phosphate buffer (pH 7.2) and 15% acetonitrile.

Partially purified samples of α-amylase inhibitor from kidney bean (Calbiochem-Behring) were used as the primary source of the α-amylase inhibitor. These samples were dissolved in water and used directly for HPLC and biological assays. Starch blockers which were previously available and marketed under the brand names of Phaseolamin (Vita Plus, Inc.), Carbo-Slim (Michigan Vitamin) and Starch Blocker (Advantage Supplements) were also used as a source of the inhibitor. Samples were prepared from 3 tablets (500 mg each) which were crushed, extracted with 100 ml of water, filtered to remove undissolved particles and assayed.

Biological activity of the inhibitor was determined by the use of Beckman Enzymatic Amylase-DS Reagent Kit (Beckman Instruments). This is an enzyme-coupled assay for α-amylase activity in which maltotetrose is the substrate and the rate of

production of NADH is followed by measuring the increase in absorbance at 340 nm. One unit of amylase activity was defined as the amount of enzyme that produced 1 μmole of NADH per minute under the conditions described below when no inhibitor was added. To assay the inhibitor, 1 ml of the Beckman reagent was preincubated at 37° C for 5 min in the spectrophotometer cuvette. At the same time another vial was preincubated and contained 750 μl of 0.05 M phosphate buffer (pH 5), 100 μl (approximately 0.03 units) of α-amylase (type I-A from porcine pancreas , Sigma Chemical Company), and inhibitor with HPLC mobile phase to give a final volume of 1100 μl. After preincubation the enzyme-inhibitor solution was transferred to the cuvette, incubated for 5 min at 37° C and the change in absorbance was measured during the next 3 min. All samples were assayed in duplicate. One unit of inhibitor was defined as the amount of inhibitor that inhibited 2 units of α-amylase by 50%.

The HPLC assay of the inhibitor was determined from a standard curve (peak height versus biological activity) which was prepared from a series of injections of the inhibitor. The equation for the standard curve was determined by linear regression analysis. Peak heights of all samples of inhibitor were calculated from the average of duplicate injections.

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RESULTS AND DISCUSSION

Separation of α-amylase inhibitor from other constituents of partially purified inhibitor and starch blocker samples was accomplished by high performance size exclusion chromatography (Figure 1). Only samples collected under peak 4 were bioactive and re-injection of the bioactive fraction on the HPLC produced a single peak with the same retention time as peak 4. In addition, when the inhibitor (1.25 units/ml) was incubated at pH 5 with α-amylase (12.5 units/ml) for 40 min at 37° C and then

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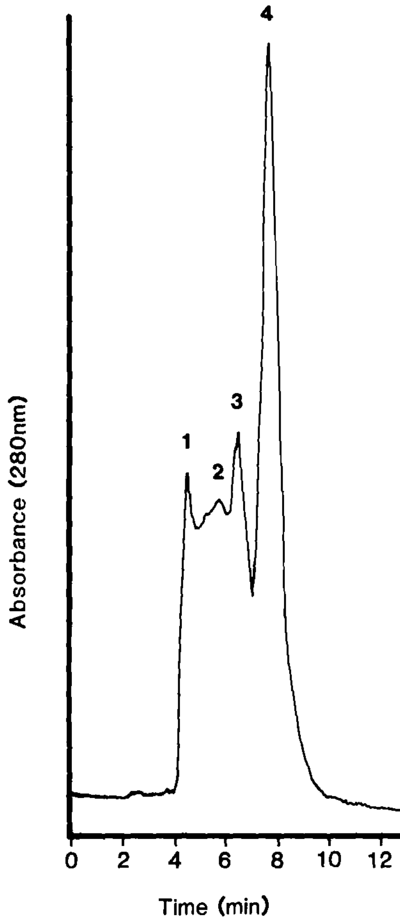

Figure 1. Chromatogram of partially purified α-amylase inhibitor using a Spherogel TSK-SW 3000 column at a flow of 1 ml/min. Injection volume was 20 μl (80 μg protein). Peak 4 contained all of the inhibitor activity.

chromatographed, a new peak appeared at 7.0 min whereas the inhibitor and α-amylase, when chromatographed separately under the same conditions, gave peaks at 7.3 and 8.8 min, respectively. The shorter retention time is indicative of a larger molecule as would be expected following the formation of an inhibitor-enzyme complex. The height of the peak at 7.0 min was also larger than either the inhibitor or amylase peaks alone.

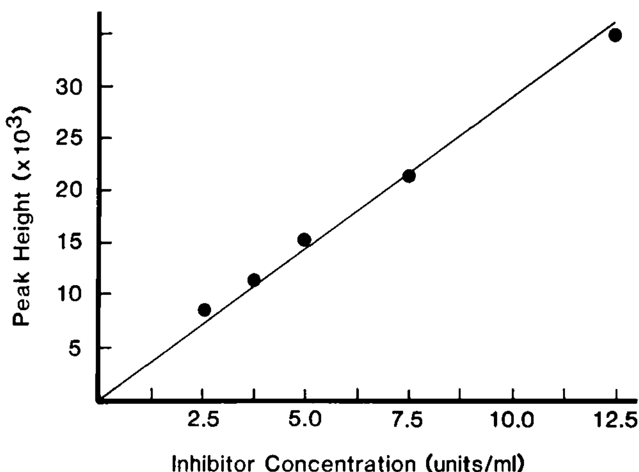

Figure 2. Standard curve for α-amylase inhibitor.

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A typical standard curve for the α-amylase inhibitor is shown in Figure 2. Peak-height and units of biological activity were linearly related over this range (correlation coefficient of 0.997). The minimal detectable quantity (sensitivity at 95% confidence) of the inhibitor by the HPLC assay was 0.13 units/ml which is comparable to the minimal detection limit (0.11 units/ml) using Beckman Enzymatic Reagent Kit.

Loss of biological activity must correspondingly alter the chromatographic response if the HPLC assay is to be useful. Table 1 illustrates that the loss of biological activity of the inhibitor correlated well with the loss of HPLC response in a sample that was dissolved in water and stored either at room temperature or at 5° C. Boiling the sample for 10 min in pH 4 phosphate buffer eliminated both biological activity and HPLC response.

Within-day and between-day precision were estimated by multiple determinations of several inhibitor solutions (Table 2). The within-day and between-day coefficients of variation were 4.8-8.9% and 3.7-11.6%, respectively. Between-day precision was difficult to study over long time periods because of the instability of the inhibitor in water at 5° C.

TABLE 1

HPLC Response And Bioassay After Inactivation Of Inhibitor

Condition	% Activity Remaining HPLC	Bioassay
Boiling water bath, 10 min	0	0
5° C for 7 days	64	66
Room temp for 2 days	70	58
Room temp for 14 days	0	0

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TABLE 2

Precision Studies

	Mean (units/ml)	SD (units/ml)	CV (%)	n
Within-day	1.66	0.08	4.8	14
	2.46	0.16	6.5	15
	10.02	0.90	8.9	15
	15.06	1.03	6.8	15
Between-day	2.35	0.13	5.5	4
	2.88	0.15	5.2	4
	4.10	0.15	3.7	4
	8.48	0.54	6.4	5
	10.11	0.86	8.5	7
	16.89	1.96	11.6	7

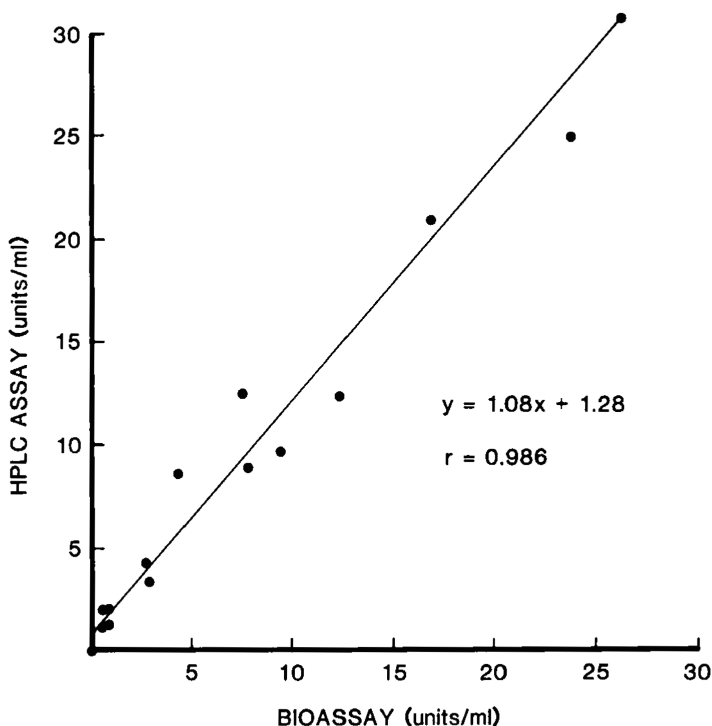

Figure 3. Comparison of HPLC assay with biological assay for α-amylase inhibitor.

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A comparison of the HPLC assay and biological assay for the amylase inhibitor is shown in Figure 3. The correlation coefficient was 0.986, indicating a good agreement between the assay methods. These assays represent different samples of partially purified inhibitor and starch blockers and not merely dilutions of such samples.

In conclusion, this HPLC assay of α-amylase inhibitor from kidney bean is simple, rapid, sensitive and reproducible. Each assay can be completed within 10 min whereas biological assay methods for the inhibitor are more time consuming and cumbersome. The HPLC response correlates well with the bioactivity even following mild inactivation of the inhibitor during storage. The

HPLC assay, however, was not evaluated for proteins that may interfere with the assay although commercially produced starch blocker tablets and samples partially purified by the procedure of Marshall (7) that we assayed did not contain interfering compounds.

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