Kidney beans, Phaseolus vulgaris, contain a proteinaceous inhibitor of α-amylase, which we have named phaseolamin. The inhibitor has been purified to homogeneity by conventional protein fractionation methods involving heat treatment, dialysis, and chromatography on DEAE-cellulose, Sephadex G-100, and CM-cellulose. Phaseolamin is specific for animal α-amylases, having no activity towards the corresponding plant, bacterial, and fungal enzymes, or any other hydrolytic enzyme tested. Optimal inhibitory activity is expressed during preincubation of enzyme and inhibitor at pH 5.5 and 37°C. Substrate prevents inhibition. Measurement of the stoichiometry of inhibition showed that a 1:1 complex of α-amylase and inhibitor is formed. Complex formation was demonstrated by chromatography on Sephadex G-100. The phaseolamin-α-amylase complex is dissociated at low pH values, apparently as a result of destruction of the enzyme; the complex cannot be dissociated by other conditions unfavorable for inhibition (low temperature or high pH). Phaseolamin inhibits hog pancreatic α-amylase in a noncompetitive manner.

Experimental Procedure

Materials

The beans from which α-amylase inhibitor was prepared (white kidney beans, Phaseolus vulgaris, variety Great Northern) were purchased from a local supermarket. α-Amylase was prepared or obtained as follows: hog pancreatic (6), from Worthington Biochemical Corp.; human salivary, by glycogen precipitation (7) from crude clarified saliva; human pancreatic, by a modification of the method of Stiefel and Keller (8); Helix pomatia, by fractionation of snail intestinal juice (Gluconase, Endo Laboratories, Garden City, N. Y.) on Sephadex G-100; Aspergillus oryzae (9), three times crystallized, from Calbiochem; Bacillus amyloliquefaciens (10), four times crystallized, sold as Bacillus subtilis (but see ref. 11) from Sigma Chemical Co.; Bacillus subtilis saccharifying α-amylase (12), from Miles Laboratories; Bacillus licheniformis, by fractionation of Thermamyl (13), from Novo Industri, Copenhagen on Sephadex G-100; rye, as described previously (14, 15); barley malt, by a procedure similar to that used for purification of rye α-amylase. Soluble starch was from Baker Chemical Co.; Sephadex G-100 and Sepharose 4B were from Pharmacia Fine Chemicals; DEAE-cellulose (DE52), and CM-cellulose (CM52), were from Whatman Biochemicals.

Methods

Measurement of α-Amylase Activity—α-Amylase activities were generally measured by the decrease of iodine staining power during action of the enzyme on soluble starch (16). Iodine staining assays were performed by using digests (total volume, 2.5 ml) containing soluble starch (10 mg), human serum albumin (1.5 mg), calcium chloride (1.5 mg), sodium glycerophosphate buffer (pH 6.9; final concentration, 20 or 40 mM) and α-amylase (up to 1 unit). During the purification steps, the inhibitor was assayed against hog pancreatic α-amylase. Activities remaining after preincubation of α-amylase and inhibitor were determined in digests containing 20 mM glycerophosphate buffer, in which enzyme action was initiated by addition of soluble starch dissolved in water. After incubation at 37°C for 5 min, samples (0.1 ml) from digests were added to solutions (5.0 ml) of 0.02% iodine in 0.2% potassium iodide, followed by measurement of absorbances at 680 nm. During studies on the properties of phaseolamin using hog pancreatic α-amylase, the procedure was similar except that enzyme action was initiated in the digests by addition of starch solution buffered with sodium glycerophosphate (pH 6.9). In this case, the final glycerophosphate buffer concentration was 40 mM to counteract the effect of the buffer.
(usually pH 5.5, vide infra) present in the preincubation mixture. The validity of the above iodometric a-amylase assay, with particular reference to its use in assay of a-amylase inhibitor activities, has been discussed elsewhere.3

Alternatively, measurements of a-amylase activity were performed by determination of reducing sugars resulting from enzyme action on soluble starch, by using an alkaline copper reagent (17). In such cases, digestion of preincubated substrate was halted by addition of 25 ml of 0.1 M sodium acetate buffer, pH 5.0, and the a-amylase activity remaining was measured as described above. Appropriate substrate blank and control digestions were run simultaneously. Under such conditions the extent of inhibition is proportional to the amount of inhibitor present, as long as the amount of inhibitor and duration of preincubation are such that not more than approximately 50% inhibition of the enzyme activity takes place. We have routinely used amounts of a-amylase inhibitor which give a valid assay, e.g., with a preincubation period of 20 min, and an a-amylase activity duration of 5 min. One unit of inhibitor activity is the amount which will bring about 50% inhibition of the a-amylase in 20 min under the above conditions. It should be noted that, although inhibitor activities have been measured on the basis of inhibitory power at pH 6.9, our studies on the properties of the purified inhibitor show that this is not the optimum pH for inhibitor action. Most studies on the properties of phaseolamin were performed by using preincubation with a-amylase at pH 5.5, followed by measurement of residual a-amylase activity at pH 6.9.

Measurement of Agglutinating Activity—Freshly obtained human erythrocytes were washed four times with a glucose/gelatin/Veronal buffer (18) and used as a suspension containing 5 x 10^11 cells/ml in this buffer. To each 25 ml of 2-fold serial dilutions of the solution being tested for agglutinating activity were added 25 ml of human erythrocyte suspension and 25 ml of 0.15 M NaCl. The mixture was shaken for 1 min and examined for agglutination after standing at 25° for 1 hour.

Other Analytical Methods—Protein was determined by the method of Lowry et al. (19) with bovine serum albumin as standard, carbohydrate by the phenol-sulfuric acid method (20) with glucose as standard, and reducing sugar measurements by reduction of alkaline copper reagent. These methods were identical to the contrary, concentrations of all phaseolamin solutions are expressed in terms of their apparent protein contents, as determined by the Lowry method.

Polyacrylamide Gel Electrophoresis—Electrophoresis was performed in polyacrylamide gels at pH 4.3 and 8.5, essentially as described by Ornstein and Davis (21, 22) but without stacking gel. Polyacrylamide gel electrophoresis under denaturing conditions (sodium dodecyl sulfate + 2-mercaptoethanol) was carried out according to Weber and Osborn (23, 24).

Dilution of Enzyme and Inhibitor Solutions—All dilutions were performed with human serum albumin solution (1 µg/ml) to minimize losses of enzyme or inhibitor caused by dissociation or by adsorption to the walls of glass test tubes (cf. Refs. 25–29). For dilution of a-amylase solutions, calcium chloride (1 mg/ml) was also present in the diluent solution to stabilize the enzyme (30).

RESULTS

Purification of Phaseolamin

Step 1: Extraction, Heat Treatment, and Dialysis—Beans (500 g) were ground to a coarse flour in a hand mill, then extracted by stirring for 1 hour at room temperature with 1,500 ml of sodium chloride solution (1%). After centrifugation (27,000 x g, 30 min), the supernatant solution was heated at 70° for 15 min in 2.5-ml portions. Coagulated protein was removed by centrifugation (27,000 x g, 60 min) and then the solution dialyzed against water at 4° overnight. After removal of precipitated material by centrifugation (27,000 x g, 30 min) the crude inhibitor preparation was dialyzed against 10 mM citrate/phosphate buffer, pH 8.0.

Step 2: Chromatography on DEAE-cellulose—After dialysis the crude inhibitor preparation was applied to a column (2.5 x 30 cm) of DEAE-cellulose equilibrated with the same buffer. Chromatography was performed at room temperature and 18-ml fractions were collected. After application of the inhibitor solution, and extensive washing with starting buffer, bound protein was recovered from the column by elution with a gradient (0 → 0.5 M, over 3 liters) of sodium chloride in 10 mM citrate/phosphate buffer. The fractions containing inhibitor activity were dialyzed against water and freeze-dried, yielding 3.82 g of partly purified inhibitor.

Step 3: Chromatography on Sephadex G-100—Partly purified, freeze-dried, inhibitor preparation (500 mg) was dissolved in 9.5 ml of 50 mM acetate buffer, pH 5.0, containing 5 mM calcium chloride, and then chromatographed at 4° on a column (90 x 2.5 cm) of Sephadex G-100 eluted with this buffer. Fractions, 5.7 ml each, were collected automatically. The fractions containing inhibitor activity were combined. In addition to the removal of contaminating proteins by molecular sieving, this step served to purify the inhibitor as a result of precipitation on the gel column of noninhibitory protein material. The precipitated protein could be removed from the column prior to reuse by washing with a slightly alkaline buffer solution, for example, Tris-HCl (50 mM, pH 7.5).

Step 4: Chromatography on CM-cellulose—The inhibitor solution from the Sephadex G-100 column was dialyzed against 10 mM acetate buffer, pH 4.0, and applied to a column (2.5 x 10 cm) of CM-cellulose equilibrated with the same buffer. Chromatography was performed at room temperature. Bound protein was recovered from the column by elution with a gradient (0 → 0.5 M) of sodium chloride in 10 mM acetate buffer, pH 4.0, 11-ml fractions being collected automatically. Inhibitor was eluted at approximately 0.2 M sodium chloride concentration, as a peak across which the specific activity of the inhibitor was almost constant. The fractions containing inhibitor were combined and dialyzed against water, and this solution was used for characterization of the properties of the inhibitor.

The results of a typical purification, resulting in a 32-fold improvement in the specific activity of the inhibitor, are presented in Table I. Examination of samples of the inhibitor prepared in this way, by electrophoresis in polyacrylamide gels at pH 4.3, showed a single protein band, indicating that the inhibitor is homogeneous. Electrophoresis at pH 8.0 provided less convincing evidence for homogeneity, a diffuse band about 0.5 cm wide being obtained with all inhibitor preparations we have made.

Alternative Purification Procedure: Isolation of Phaseolamin with Immobilized a-Amylase—As an alternative to the above conventional fractionation scheme, small amounts of α-amylase inhibitor may be isolated from crude extracts or partly purified inhibitor preparations, by use of a-amylase covalently bound to Sepharose 4B.

1 This was prepared by using 0.1 M citric acid and 0.2 M disodium hydrogen phosphate solutions (31) and then diluted 10-fold.
Table I

<table>
<thead>
<tr>
<th>Steps and procedures</th>
<th>Total units</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>Purification</th>
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<tr>
<td>Step 1: Crude extract (500 g of beans)</td>
<td>312,800</td>
<td>64,600</td>
<td>4.8</td>
<td>100</td>
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<td>Step 2: Heat treatment</td>
<td>222,600</td>
<td>34,000</td>
<td>6.5</td>
<td>71</td>
<td>1.4</td>
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<tr>
<td>Step 3: Dialysis</td>
<td>203,200</td>
<td>10,100</td>
<td>20.1</td>
<td>65</td>
<td>4.2</td>
</tr>
<tr>
<td>Step 4: DEAE-cellulose chromatography</td>
<td>142,500</td>
<td>3,100</td>
<td>66</td>
<td>46</td>
<td>8.6</td>
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<tr>
<td>Step 5: Sephadex G-100 chromatography*</td>
<td>150,100</td>
<td>1,150</td>
<td>131</td>
<td>48</td>
<td>27.3</td>
</tr>
<tr>
<td>Step 6: CM-cellulose chromatography*</td>
<td>116,700</td>
<td>770</td>
<td>152</td>
<td>37</td>
<td>31.7</td>
</tr>
</tbody>
</table>

*Steps 5 and 6 were performed on only a part of the material obtained from Step 4. However, the data for Steps 5 and 6 are presented as if all the material had been subjected to the last two purification steps.

Coupling of α-amylase to Sepharose 4B was performed essentially as described by Porath et al. (32). The insoluble Sepharose-α-amylase conjugate was stored in 20 mM acetate buffer, pH 5.5, containing 4 mM calcium chloride, and portions were removed from the suspension as needed, by centrifugation. Inhibitor preparations dissolved in, or dialyzed against, 20 mM acetate buffer, pH 5.5, containing 4 mM calcium chloride, were added, mixed well, and incubated with the immobilized α-amylase at 37 °C for 30 min with occasional shaking. After incubation, the gel was removed from the suspension by centrifugation and washed with buffer. α-Amylase inhibitor was recovered from its complex with the bound α-amylase by washing with 100 mM citrate/phosphate buffer, pH 3.1. Preparations of inhibitor made in this way had specific activities similar to those prepared by the conventional procedures described above. However, the method is only suitable for the isolation of small quantities of inhibitor.

Properties of Phaseolamin

Molecular Weight and Subunit Structure—The molecular weight determined by gel chromatography on a calibrated column (86 x 2.5 cm) of Sephadex G-200 (33) was 49,000 (Fig. 1a). On chromatography on a column (90 x 1.5 cm) of Sephadex G-100 (Fig. 1b) the inhibitor emerged at an elution volume slightly lower than that of ovalbumin (molecular weight, 43,500). Thus the indications are that phaseolamin is a protein with molecular weight in the range 45,000 to 50,000. The value of 49,000 obtained from the calibrated G-200 column, has been used for calculation of molarities of phaseolamin solutions.

Freedom from Agglutinating Activity—When a solution of phaseolamin with a concentration of 1.08 mg/ml was tested, agglutination of human red blood cells did not take place. Under the same conditions agglutination was caused by a crude extract of kidney beans of concentration 0.07 mg/ml or greater.

Analysis for Carbohydrate—Determination of total phenolsulfuric acid-positive carbohydrate indicated that phaseolamin is a glycoprotein containing 9 to 10% carbohydrate. The nature of the monosaccharide constituents was not investigated.

Heat Inactivation of Phaseolamin—A solution of phaseolamin with a concentration of 0.075 mg/ml in 1.5 mM acetate buffer, pH 4.0, was placed in a sealed tube and heated at 100°. Samples were removed for assay of residual inhibitor activity after heating for 10, 20, and 30 min. At each time, samples were also transferred to an ice bath and assayed after standing at 0° for 2 hours. Inhibitory activity was found to have been completely destroyed after 10 min at 100°; no recovery of activity took place during storage at 0° for 2 hours.

Ethanol Solubility of Phaseolamin—The solubility of phaseolamin in 70% ethanol was determined after suspending 3.0 mg of the freeze-dried solid in 3.0 ml of 70% ethanol, standing at room temperature, and centrifuging. Based on the inhibitory activity of the supernatant solution compared with that of a solution prepared by dissolving 3.0 mg of the freeze-dried solid in water, the solubility in 70% ethanol was found to be only 10% of the solubility in water at the same concentration. The low solubility of phaseolamin in aqueous ethanol was confirmed during attempts to extract the inhibitor from bean flour with 70% ethanol, which failed to extract significant amounts of the material.

Inhibition of Hog Pancreatic α-Amylase by Phaseolamin; Time and Temperature Dependence—Phaseolamin (6.75 μg of protein) was preincubated at pH 6.9 with α-amylase (1 unit) in digest media: 0.1, 0.05 M citrate buffer, pH 5.5, containing 4 mM calcium chloride and 20 mM acetic acid.
The activity remaining at each time was determined by the iodine staining assay. This experiment was performed with preincubation at 0, 25, and 37. The results for 25 and 37°C are shown in Fig. 2. No measurable inhibition took place at 0°C.

Effect of pH on Inhibitory Activity—The extent of inhibition of hog pancreatic α-amylase was determined at different pH values by preincubation of α-amylase (1 unit) with inhibitor (1.76 µg of protein) in digests of the usual composition (see “Experimental Procedure”), but containing buffers (citrate/phosphate, final concentration, 6.7 mM) of various pH values. The activity remaining after preincubation at 37°C for 20 min, was determined by the iodine staining assay. Control digests were included to correct for the loss of α-amylase activity during preincubation, which is considerable at the lower pH values. Fig. 3 shows the effect of pH on inhibitory power, the inhibition being expressed as the total decrease in α-amylase activity during preincubation with inhibitor, less the amount of activity lost by preincubation of the α-amylase at a given pH in the absence of inhibitor.

Reversibility of Inhibition—Attempts were made to determine whether the conditions found to be unfavorable for inhibition, namely high pH and low temperature, will reverse inhibition which has already taken place.

To test the effect of pH, the extent of inhibition of α-amylase was measured by the iodine staining assay after preincubation (37°C, 30 min) of α-amylase (1 unit) with inhibitor (8.8 µg) in digests (1.5 ml) of the usual composition but containing acetate buffer, pH 5.5, with a concentration of 1.3 mM. The extent of inhibition of α-amylase was also measured after preincubation for 30 min as above, followed by a further preincubation for 30 min at pH 8.2, the pH being adjusted to this value by addition of disodium hydrogen phosphate solution (0.1 ml, 0.2 M). In each case, the amylase activity was measured by addition of soluble starch solution buffered at pH 6.9 and removal of a sample for iodine staining measurements.

Activities were compared with those in appropriate control digests from which the α-amylase inhibitor was omitted. When preincubation at pH 5.5 was followed by preincubation at pH 8.2, the measured α-amylase activity was identical to that after preincubation only at pH 5.5. Thus, while elevation of the pH prevents further inhibition, it does not reverse inhibition which has already occurred.

To test the effect of low temperature, the extent of inhibition of α-amylase was measured by the iodine staining assay after preincubation (180 min) of α-amylase (1 unit) with inhibitor (0.88 µg) in digests (1.5 ml) of the usual composition buffered with 6.7 mM acetate, pH 5.5, and after preincubation of an identical digest under the same conditions for the same length of time, followed by 45 min at 0°C. The amount of inhibition which took place in both digests was identical (51%).

Effect of Substrate on Interaction of α-Amylase and Inhibitor—To determine whether inhibitor can interact with α-amylase in the presence of substrate, or whether substrate can dissociate the enzyme-inhibitor complex, digests were prepared in which (a) α-amylase was allowed to degrade starch in the absence of inhibitor, (b) substrate was added to a mixture of α-amylase and inhibitor, and (c) inhibitor was added to a mixture of α-amylase and substrate. In each case the amount of α-amylase used was approximately 1 unit, and in Digests b and c the amount of inhibitor was 8.8 µg. The digests, which were incubated at 37°C, contained 1.5 mg of human serum albumin, 1.5 mg of calcium chloride, 10 mg of soluble starch, and 40 mM sodium glycerophosphate buffer, in a total volume of 2.5 ml. In Digest b, starch solution buffered at pH 6.9 was added after preincubation of the other digest constituents at 37°C for 30 min. In Digest c inhibitor was added last, immediately after addition of α-amylase to the other digest constituents. Samples (0.1 ml) were removed from each digest at intervals for iodine staining measurements. The results are shown in Fig. 4.

![Fig. 2 (left). The time course of inhibition of hog pancreatic α-amylase by purified phaseolamin at 37 and 25°C. Phaseolamin (0.75 µg of protein) was preincubated at each temperature with α-amylase (1 unit) in digests (1.5 ml) containing human serum albumin (1.5 mg) and calcium chloride (1.5 mg) buffered with sodium glycerophosphate (35.3 mM, pH 6.9). After different times, residual α-amylase activity was determined by using the iodine staining assay.

Fig. 3 (center). The pH dependence of the inhibitory activity of phaseolamin towards hog pancreatic α-amylase. Digests (1.5 ml) containing α-amylase (1 unit), phaseolamin (1.76 µg of protein), human serum albumin (1.5 mg), calcium chloride (1.5 mg), and buffers of different pH values (citrate/phosphate, final concentration, 6.7 mM) were preincubated at 37°C for 20 min. Residual α-amylase activity then was determined by using the iodine staining assay, after addition of soluble starch solution (1.0 ml) buffered with sodium glycerophosphate, pH 6.9.

Fig. 4 (right). Degradation of starch by hog pancreatic α-amylase. •, the decrease of iodine staining power of starch by α-amylase alone; O, the decrease of iodine staining power after addition of phaseolamin to a mixture of enzyme and substrate; ◼, the decrease of iodine staining power after addition of substrate to a mixture of phaseolamin and α-amylase. In all cases the final composition of the digests (volume, 2.5 ml) was: hog pancreatic α-amylase, 1 unit; human serum albumin, 1.5 mg; calcium chloride, 1.5 mg; soluble starch, 10 mg; sodium glycerophosphate buffer (pH 6.9), 40 mM; and phaseolamin (when present), 8.8 µg. The digests were incubated at 37°C and samples (0.1 ml) were removed at intervals for iodine staining measurements.
Stoichiometry of Inhibition—The stoichiometry of inhibitor-amylase interaction was investigated by preincubation of digests containing accurately determined amounts of hog pancreatic α-amylase and phaseolamin, for different periods of time, to determine the maximum extent of inhibition. The preincubation was performed at 37° in digests (1.5 ml) containing acetate buffer (pH 5.5, 6.7 mM), human serum albumin (1.5 mg), calcium chloride (1.5 mg), α-amylase (0.81 μg), and phaseolamin (0.4 μg). The amount of α-amylase used was determined by using a molar extinction coefficient at 280 nm of 12.8 x 10^4 (6), based on a molecular weight of 51,300 (34); the amount of phaseolamin was measured on a total dry weight basis. The α-amylase activity remaining after the different times of preincubation was determined in the usual manner, by addition of buffered starch solution and removal of samples for iodine staining measurements.

The maximum extent of inhibition observed (41.5%, Fig. 5) corresponds to inhibition of 0.34 μg of α-amylase. Thus, 0.34 μg of α-amylase is inhibited by 0.40 μg of inhibitor and, by using values for the molecular weights of α-amylase as 51,300 (34) and that of the inhibitor as 49,000 it is found that 8.16 x 10^-4 μmol of inhibitor rendered inactive 6.63 x 10^-8 μmol of α-amylase. This calculation assumes that all the protein in the α-amylase preparation is active enzyme; and would be in error if any inactive α-amylase, with which the inhibitor cannot bind, were present.

Demonstration of Complex Formation between Phaseolamin and Hog Pancreatic α-Amylase—A solution of hog pancreatic α-amylase containing approximately 2 mg of enzyme was prepared by centrifuging an appropriate volume of the crystalline suspension, followed by addition of 0.5 ml of 50 mM acetate buffer, pH 5.0, containing 5 mM calcium chloride, to the precipitated enzyme and warming at 37° to dissolve. After centrifugation to remove insoluble material, α-amylase inhibitor (2.0 ml of a solution containing 1.76 mg of protein) was added and the mixture was incubated at 37° for 2.5 hours. The α-amylase/inhibitor mixture was then chromatographed at room temperature on a column (85 x 1.5 cm) of Sephadex G-100 and eluted with 50 mM acetate buffer, pH 5.0, containing 10 mM sodium chloride.

Fractions, 3.4 ml each, were collected automatically, and the distribution of protein in the column effluent was determined by measurement of the absorbance of fractions at 280 nm. α-Amylase and phaseolamin, in amounts corresponding to that present in the mixture, were also chromatographed separately on the same column. The results are shown in Fig. 6. It should be noted that, although hog pancreatic α-amylase and phaseolamin have approximately the same molecular weight, their positions of elution from the Sephadex column differ markedly. This is because hog pancreatic α-amylase is retarded as a result of an enzyme-substrate type of affinity for the polyacrylamide gel matrix (35).

Specificity of Phaseolamin—The purified α-amylase inhibitor was tested for its ability to inhibit α-amylases from a number of sources, plant, animal, fungal, and bacterial. Inhibitor (8.8 μg of protein) was preincubated with enzyme (0.05 unit) in digests (0.5 ml) containing buffer (10 mM acetate, pH 5.0), human serum albumin (0.75 mg), and calcium chloride (0.75 mg). After 30 min at 37°, the activity remaining was determined by addition of substrate (10 mg/ml, 0.5 ml) and incubation at 37° for 15 min and measurement of the amount of reducing sugars produced. The extents of inhibition of the different α-amylases (Table II) are expressed relative to the activities in control digests preincubated without α-amylase.

Table II

<table>
<thead>
<tr>
<th>Source of α-amylase</th>
<th>Extent of inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus amyloquefaciens</td>
<td>2</td>
</tr>
<tr>
<td>Bacillus subtilis (saccharifying amylase)</td>
<td>0</td>
</tr>
<tr>
<td>Bacillus licheniformis</td>
<td>0</td>
</tr>
<tr>
<td>Aspergillus oryzae</td>
<td>3</td>
</tr>
<tr>
<td>Barley malt</td>
<td>0</td>
</tr>
<tr>
<td>Rye</td>
<td>0</td>
</tr>
<tr>
<td>Helix pomatia</td>
<td>97</td>
</tr>
<tr>
<td>Hog pancreas</td>
<td>97</td>
</tr>
<tr>
<td>Human saliva</td>
<td>94</td>
</tr>
<tr>
<td>Human pancreas</td>
<td>100</td>
</tr>
</tbody>
</table>

*Results are expressed as per cent of activity in controls without α-amylase inhibitor. Details are given under "Results."

![Fig. 5 (left). The determination of the stoichiometry of phaseolamin-hog pancreatic α-amylase interaction. Phaseolamin (0.40 μg, 8.16 x 10^-4 μmol) was incubated at 37° with a 2-fold molar excess (0.81 μg, 16.0 x 10^-5 μmol) of hog pancreatic α-amylase. In digests (1.5 ml) containing acetate buffer (pH 5.5, 6.7 mM), human serum albumin (1.5 mg), and calcium chloride (1.5 mg), and after different durations of preincubation the residual α-amylase activity was determined by using the iodine staining assay, until maximum inhibition was achieved.](http://www.jbc.org/)
lase inhibitor. When tested at similar concentrations, phaseolamin was found to be without inhibitory effect on sweet potato $\alpha$-amylase, rabbit muscle acid and neutral $\alpha$-glucosidases, and bovine pancreatic trypsin.

The rates of inhibition of hog pancreatic, salivary, and Helix pomatia $\alpha$-amylases were measured by preincubation of $\alpha$-amylase inhibitor (4.4 $\mu$g) for various periods of time with samples (approximately 1 unit) of each $\alpha$-amylase in digests of the same composition as used for the assay of inhibitor activity but buffered with 6.7 mM acetate, pH 5.5. After preincubation for different lengths of time, the activity remaining was determined by the iodiode staining assay after addition of appropriately buffered substrate solution (1.0 ml, pH 6.9) for salivary and hog pancreatic $\alpha$-amylases and pH 5.5 for the $H$. pomatia enzyme. Hog pancreatic and salivary $\alpha$-amylases were inhibited at identical rates; the rate of inhibition of $H$. pomatia $\alpha$-amylase was approximately half that of the other two $\alpha$-amylases.

Nature of $\alpha$ Amylase Inhibition by Phaseolamin To ascertain whether inhibition of hog pancreatic $\alpha$-amylase by phaseolamin was competitive or noncompetitive, Lineweaver-Burk plots were drawn for the uninhibited and partially inhibited enzyme. The rate of $\alpha$-amylase action was determined at different substrate concentrations in digests (1.0 ml) containing soluble starch (0.5, 1.25, and 2.5 mg/ml), human serum albumin (0.15 mg), calcium chloride (0.15 mg), sodium glycerophosphate buffer (50 mM, pH 6.9), and enzyme (25 $\mu$l of a solution containing 0.9 unit/ml). The rate of $\alpha$-amylase action also was determined after preincubation (37°C, 1 hour) of the same amount of enzyme with inhibitor (0.064 $\mu$g of protein), human serum albumin (0.15 mg), calcium chloride (0.15 mg), and acetate buffer (pH 5.5, 10 mM) in a volume of 0.5 ml, followed by addition of starch solution (0.5 ml, concentrations of 1.0, 2.5, and 5.0 mg/ml) buffered with 100 mM sodium glycerophosphate buffer pH 6.9. The Lineweaver-Burk double reciprocal plots for the uninhibited and partly inhibited enzyme intersected on the abscissa (Fig. 7), indicating that phaseolamin is a noncompetitive inhibitor of hog pancreatic $\alpha$-amylase. This treatment assumes that enzyme-inhibitor interaction is not irreversible.

**DISCUSSION**

Earlier reports (2, 3, 5) have indicated that specific proteinaceous inhibitors of $\alpha$-amylase are widespread in kidney beans (Phaseolus vulgaris) and other legumes. Since $\alpha$-amylase plays an important role in starch breakdown in human beings and animals, the presence of such inhibitors in foodstuffs may be responsible for impaired starch digestion (1, 4). $\alpha$-Amylase inhibitors may be of value as novel therapeutic and dietetic agents (1, 36). *P. vulgaris* contains a number of other toxic agents (1, 36). While phaseolamin is also rather heat-stable, heat treatment at a temperature of 70°C being used in its purification, it is, unlike the wheat inhibitors, rapidly destroyed on heating at 100°C. Reactivation of the heat-inactivated inhibitor could not be demonstrated.

Other workers have reported the purification of $\alpha$-amylase inhibitors from wheat (43), but neither form of the inhibitor was reported to be glycoprotein in nature. A further difference between phaseolamin and the wheat inhibitors is the solubility in ethanolic solution. The wheat inhibitors may be extracted from flour with 70% ethanol (44-46). Phaseolamin could not be extracted from ground beans in this way; neither was the purified inhibitor significantly soluble in this concentration of ethanol. Wheat $\alpha$-amylase inhibitors are heat-stable proteins (1, 43, 44). While phaseolamin is also rather heat-stable, heat treatment at a temperature of 70°C being used in its purification, it is, unlike the wheat inhibitors, rapidly destroyed on heating at 100°C. Reactivation of the heat-inactivated inhibitor could not be demonstrated.

Studies on the optimum conditions for inhibition of hog pancreatic $\alpha$-amylase by phaseolamin have confirmed and extended our previous findings. 1 Inhibition of the amylase is dependent on the duration, pH, and temperature of interaction of amylase and inhibitor. The optimum pH for inhibition is at about pH 5.5, and inhibition takes place much more rapidly at 37°C than at 0 or 25°C. The inhibition of $\alpha$-amylase by phaseolamin cannot, however, be reversed by adjustment to conditions which are unfavorable for inhibition. Thus, decrease of temper-
ature, or increase of pH, did not reverse inhibition of hog pancreatic α-amylase. Neither does substrate reverse inhibition, although phaseolamin does not cause any measurable inhibition of α-amylase in the presence of substrate, presumably because of the high affinity of the α-amylase for its substrate, present in large excess.

The stoichiometry of hog pancreatic α-amylase-phaseolamin interaction was investigated by measurement of the maximum amount of α-amylase which a known amount of the inhibitor could render inactive. For this experiment α-amylase was quantitated by its ultraviolet absorbance and phaseolamin was measured on a dry weight basis. Inhibition of $6.63 \times 10^{-4}$ mol of α-amylase was caused by $8.16 \times 10^{-4}$ mol of the inhibitor. Similar results were obtained when different amounts of hog pancreatic α-amylase were treated with the same amount of phaseolamin. These figures indicate that a 1:1 complex of amylase and inhibitor is formed.

Other than the apparent 1:1 interaction of the amylase and inhibitor, the results which were obtained could be explained on the basis of proteolytic inactivation of α-amylase, rather than by the formation of a specific enzyme inhibitor complex as takes place during interaction of proteolytic enzymes with protease inhibitors (47, 48). However, this alternative was ruled out, and the specific interaction of phaseolamin with hog pancreatic α-amylase was demonstrated definitively by gel filtration of the soluble complex produced on interaction of α-amylase with phaseolamin. After mixing approximately equimolar amounts of hog pancreatic α-amylase and phaseolamin, a complex is produced which emerges from a Sephadex column at a point consistent with its having the expected molecular weight of about 100,000 (Fig. 6). Thus, phaseolamin is a specific α-amylase inhibitor rather than a proteolytic enzyme. This same experiment also indicates that phaseolamin does not act by chelating calcium or removal of chloride, these ions being required for the stability and activity of α-amylase.

Like most other α-amylase inhibitors which have been investigated (1), phaseolamin is specific for animal α-amylases. Human salivary and pancreatic, hog pancreatic, and H. pomatia α-amylase are the only enzymes which have been found to be inhibited to significant extents. The activity of plant and microbial α-amylases is unaffected by phaseolamin, although the possibility of complex formation between these enzymes and the inhibitor has not yet been ruled out. We have been unable to confirm the claim (5) that P. vulgaris α-amylase inhibit bacterial α-amylase. Rather, we explain the reported loss of activity of the latter enzyme, during preincubation with inhibitor, as being spontaneous inactivation during preincubation, presumably caused by traces of proteolytic enzymes which are present even in crystalline preparations of the bacterial enzyme (30).

It is interesting to speculate regarding the in vivo function of phaseolamin. Since this inhibitor is without effect on plant α-amylases, it is unlikely that it serves to regulate the activity of kidney bean α-amylase. An alternative function, that of protecting the carbohydrate reserves against microbial degradation, is also unlikely since microbial α-amylases are not inhibited by phaseolamin. The most likely function for phaseolamin is that it serves as a protective agent against insects and other predators by virtue of its ability to inhibit their digestive amylases.

Further studies are in progress to determine the mechanism of the inhibitory action of phaseolamin, and to examine its physiological effect in vivo.

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