

### 2.3. Measurement of $\alpha$ -Amylase Activities

The activities of stock salivary and pancreatic  $\alpha$ -amylase solutions were determined by measurement of the reducing sugars produced during action on soluble starch, by using a copper-reducing power method [5]. Full details have been given previously [3].

Measurement of  $\alpha$ -amylase inhibitor activity depends on determination of  $\alpha$ -amylase activity remaining after interaction of an aliquot of inhibitor preparation with a known amount of  $\alpha$ -amylase. For this procedure, a modification of an iodine staining power assay of  $\alpha$ -amylase activity was used [6]. To a solution (1.5 ml, this being the pre-incubation digest, *vide infra*) containing up to 1 Unit of  $\alpha$ -amylase, at 37 °C, is added a solution (1.0 ml) of soluble starch solution (10 mg/ml in 100 mM glycerophosphate buffer pH 6.9), which has been pre-warmed to 37 °C, and mixed. After incubation at 37 °C for exactly 5 min, an aliquot (0.1 ml) from the digest is added to a solution (5.0 ml) of 0.02% iodine in 0.2% potassium iodide, and the absorbance of the resulting starch-iodine complex read at 680 nm. The  $\alpha$ -amylase activity is expressed as the change in absorbance from that of a control digest without  $\alpha$ -amylase. Control experiments showed that the decrease in absorbance is directly proportional to the  $\alpha$ -amylase activity in the digest as long as the decrease in absorbance does not exceed 65%.

### 2.4. General Considerations Regarding $\alpha$ -Amylase Inhibitor Assay

**2.4.1. Selection of  $\alpha$ -amylase.** Studies on the specificity of phaseolamin showed that, like most other  $\alpha$ -amylase inhibitors which have been examined, its inhibitory activity is restricted to  $\alpha$ -amylases from animal sources, e.g. pancreatic  $\alpha$ -amylase and salivary  $\alpha$ -amylases [3]. Microbial and plant  $\alpha$ -amylases are, therefore, unsuitable for use in the assay of  $\alpha$ -amylase inhibitor activity.

**2.4.2. Spontaneous loss of  $\alpha$ -amylase inhibitor activity.** The action of phaseolamin on  $\alpha$ -amylase is not instantaneous; assay of inhibitor activity requires a pre-incubation step (*vide infra*) during which amylase and inhibitor interact. Preliminary experiments indicated that, in addition to inhibition of  $\alpha$ -amylase activity, spontaneous loss of enzyme activity also took place during the pre-incubation period. This was particularly troublesome in the case of hog pancreatic  $\alpha$ -amylase and is presumably due, at least in part, to the action of proteolytic enzymes, which are usually present in even the most highly purified preparations of pancreatic  $\alpha$ -amylase [7]. The problem could be conveniently circumvented by incorporation of calcium chloride and human serum albumin, both at a concentration of 1 mg/ml, into the pre-incubation digest. Under these conditions both hog pancreatic and human salivary  $\alpha$ -amylases retained 100% activity during pre-incubation at 37 °C, in the absence of inhibitor. Neither calcium chloride nor human serum albumin alone was completely successful in preventing loss of activity.

**2.4.3. Interference by amylolytic enzymes in seed extracts.** The  $\alpha$ -amylase inhibitor assay procedure (*vide infra*) involves determination of the amount of inhibition of a fixed amount of  $\alpha$ -amylase, caused by an aliquot of inhibitor preparation. When the inhibitor preparation itself contains

amylolytic enzymes, the results obtained will be inaccurate because of the effect of the endogenous amylases on the iodine staining power of the substrate. Suitable control digests are necessary in such cases. The decrease in iodine stain of the substrate caused by the inhibitor preparation alone is determined and subtracted from the decrease in iodine stain caused by the uninhibited amylase in the test digest. It is advisable to include such controls when crude extracts are being assayed, although the amount of endogenous amylase in legume extracts is low, and its effect on the assay is small.

**2.4.4. Principle of inhibitor assay.** Two types of inhibitor assay are possible. The first would involve measurement of the maximum amount of  $\alpha$ -amylase which an aliquot of inhibitor preparation can render inactive, this being determined directly by the stoichiometry of  $\alpha$ -amylase:inhibitor interaction. The alternative method would involve measurement of an extent of inhibition which is less than the maximum obtainable with the aliquot used, and expressing the inhibitor activity in terms of the rate of inhibition of a fixed amount of  $\alpha$ -amylase.

Assays based on determination of the total amount of  $\alpha$ -amylase which a sample of the inhibitor can render inactive were unsatisfactory, the main problem being the requirement for extended pre-incubation times to achieve maximum inhibition, and the resulting complications associated with spontaneous loss of  $\alpha$ -amylase activity. Measurement of the rate of inhibition of  $\alpha$ -amylase was found to be much more convenient and accurate, requiring only short pre-incubation times, although these times must be carefully controlled.

## 3. Results

### 3.1. Dependence of Inhibitor Activity on Time and Temperature of Pre-incubation

The extent of inhibition of a fixed amount of  $\alpha$ -amylase by a certain amount of phaseolamin is dependent on the duration of pre-incubation. Figure 1 shows the time course of

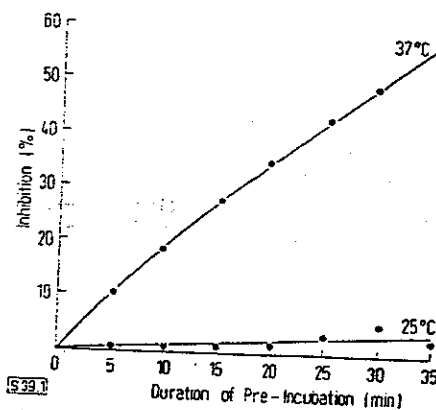


Figure 1. The time course of inhibition of hog pancreatic  $\alpha$ -amylase by purified phaseolamin at 25 °C and 37 °C. Phaseolamin (6.75  $\mu$ g protein) was preincubated at each temperature with hog pancreatic  $\alpha$ -amylase (1 Unit) in digests (1.5 ml) containing human serum albumin (1.5 mg) and calcium chloride (1.5 mg), buffered with sodium glycerophosphate (33.5 mM, pH 6.9). After different times, residual  $\alpha$ -amylase activity was determined by using the iodine staining assay.

inhibition at temperatures of 25 °C and 37 °C. Inhibition did not take place when  $\alpha$ -amylase and inhibitor were pre-incubated at 0 °C. The extent of inhibition at 37 °C is approximately proportional to the duration of pre-incubation up to 55% inhibition. It should be noted that in this experiment the pH of the pre-incubation digest (6.9) was not the optimum pH for inhibitor activity (*vide infra*).

### 3.2. Proportionality between Extent of Inhibition and Amount of Inhibitor Used

A fixed amount of  $\alpha$ -amylase was pre-incubated with different known amounts of phaseolamin for 20 min at 37 °C. The amount of  $\alpha$ -amylase remaining in each digest was then determined. The results, shown in Figure 2, show that under the conditions used there is a linear relationship between the extent of inhibition and the amount of inhibitor preparation incorporated into the pre-incubation digest, as long as the extent of inhibition does not exceed 50%. As in the experiment above, the pH of the pre-incubation digest was 6.9, which does not result in maximal inhibitor activity.

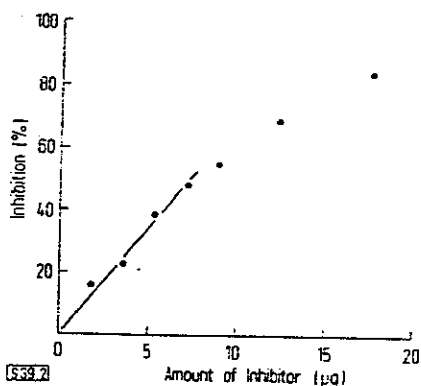


Figure 2. Inhibition of  $\alpha$ -amylase by different amounts of phaseolamin. Digests (1.5 ml) containing hog pancreatic  $\alpha$ -amylase (1 Unit), phaseolamin (1.76–17.6  $\mu$ g protein), human serum albumin (1.5 mg) and calcium chloride (1.5 mg), buffered with sodium glycerophosphate (33.3 mM, pH 6.9) were pre-incubated at 37 °C for 20 min. The residual  $\alpha$ -amylase activity was then determined by using the iodine staining assay.

### 3.3. Dependence of Inhibitor Activity on pH of Pre-incubation

$\alpha$ -Amylase was pre-incubated at 37 °C with phaseolamin at a number of different pH values. Following pre-incubation for 20 min, residual  $\alpha$ -amylase activity was determined by addition of soluble starch solution (1.0 ml, buffered with 100 mM sodium glycerophosphate pH 6.9) to each pre-incubation digest, and use of the iodine staining assay as described above. The effect of the pH of pre-incubation on the extent of inhibition is shown in Figure 3.

### 3.4. Protocol for Measurement of $\alpha$ -Amylase Inhibitor Activities

Taking into consideration the above observations, the following procedure for determination of inhibitor activity was developed.

An appropriate amount of inhibitor solution is pre-incubated at 37 °C with  $\alpha$ -amylase (hog pancreatic or human salivary, approximately 1  $\mu$ g containing about 1 Unit of

$\alpha$ -amylase activity), human serum albumin (1.5 mg) and calcium chloride (1.5 mg) in 33.3 mM acetate buffer pH 5.5, the volume of the pre-incubation mixture being 1.5 ml. After a suitable duration of pre-incubation, starch solution (1.0 ml, 10 mg/ml in 100 mM sodium glycerophosphate buffer

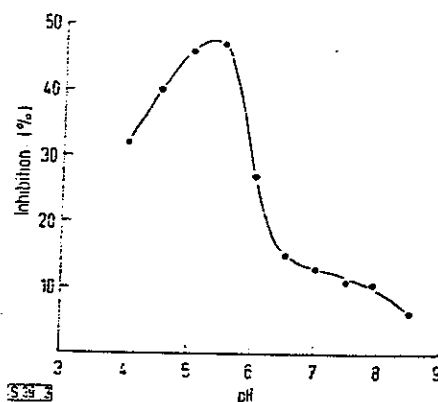


Figure 3. The pH dependence of the inhibitory activity of phaseolamin towards hog pancreatic  $\alpha$ -amylase. Digests (1.5 ml) containing  $\alpha$ -amylase (1 Unit), phaseolamin (1.76  $\mu$ g protein), human serum albumin (1.5 mg), calcium chloride (1.5 mg) and buffers of different pH values (citrate-phosphate, final concentration 6.7 mM) were pre-incubated at 37 °C for 20 min. Residual  $\alpha$ -amylase was then determined by using the iodine staining assay, after addition of soluble starch (1.0 ml) buffered with 100 mM sodium glycerophosphate pH 6.9. Control digests were included to correct for the loss of  $\alpha$ -amylase activity during pre-incubation, which is considerable at the lower pH values. The inhibition is expressed as the total decrease in  $\alpha$ -amylase activity during pre-incubation with inhibitor, less the amount of activity lost by pre-incubation of the  $\alpha$ -amylase at the same pH in the absence of inhibitor.

pH 6.9) is added and the  $\alpha$ -amylase activity remaining measured by the decrease in iodine stain during incubation for 5 min. Appropriate substrate blank and control digests are run simultaneously. One unit of  $\alpha$ -amylase inhibitor activity has been defined as the amount which causes 50% inhibition of the  $\alpha$ -amylase in 20 min under the above conditions.

The amount of  $\alpha$ -amylase used in each digest should be such that the uninhibited enzyme gives a decrease in iodine staining power of the substrate which is in the range where it is linear with time (65% decrease). The amount of inhibitor and duration of pre-incubation should be such that not more than approximately 50% inhibition of the enzyme takes place. The validity of the assay procedure under these conditions is apparent from Figures 1 and 2. Thus Figure 2 shows the direct proportionality between the amount of inhibitor and the extent of inhibition. Figure 1, likewise, shows that the extent of inhibition at 37 °C is approximately proportional to the duration of pre-incubation up to 50% inhibition. The slight deviation from linearity in the latter case (Fig. 1) is probably accountable for by inhibition taking place during the 5 min  $\alpha$ -amylase assay period, which follows the pre-incubation. Closer linearity can be obtained by considering the time of interaction of  $\alpha$ -amylase and inhibitor as the pre-incubation time plus half the assay duration (2.5 min). However, throughout this work, we have used pre-incubation times which are relatively long (usually 20 min) compared with the assay time

15 min; and the effect of assuming that further inhibition does not take place during the  $\alpha$ -amylase assay does not introduce significant error. In any case, it should be noted that the experiment illustrated in Figure 1 was performed with both pre-incubation and assay at pH 6.9; when pre-incubation is performed at pH 5.5 less inhibitor will be present, and not significant amount of inhibition will occur during the assay process at pH 6.9.

### 3.3. Measurement of $\alpha$ -Amylase Inhibitor Activity in Legume Extracts

Seeds to be tested were ground to a coarse flour in a hand mill. Samples (25 g) were then extracted with 100 ml water for 1 h at room temperature with stirring. The extracts were then filtered through cheesecloth, and clarified by centrifugation (30 min, room temperature, 12,000 g). The clear supernatant solutions were then assayed for inhibitor activity towards salivary and pancreatic  $\alpha$ -amylases as described above. The results are shown in Table 1.

Table 1.  $\alpha$ -Amylase Inhibitor Activity in Varieties of *Phaseolus vulgaris*.

Variety	Inhibitor activity			
	Pancreatic $\alpha$ -amylase		Salivary $\alpha$ -amylase	
	Units/g beans	Units/mg protein	Units/g beans	Units/mg protein
White kidney beans (Great Northern)	5040	56	4540	63
Navy beans	6680	43	6552	44
all white beans	3690	40	3610	41
ck beans	5310	45	5170	47
Pinto beans	4560	41	4400	45
Red kidney beans	4780	48	4080	57
Small red beans (chili beans)	2850	42	2900	41
Pink beans	2860	74	3020	70

Extracts of the beans were prepared and assayed against salivary and pancreatic  $\alpha$ -amylases as described in the text. Inhibitor levels are expressed in terms of total units/g of dry beans, and as the specific activity (units/mg protein) in the extract.  $\alpha$ -Amylase inhibitor activity was absent in extracts of the following legumes: lima beans, garbanzo beans, black eye peas, lentils, split peas, whole peas, soy beans, Jack beans and mung beans.

## 4. Discussion

Proteinaceous inhibitors of  $\alpha$ -amylase are widespread in plants [1]. A variety of methods for determination of  $\alpha$ -amylase inhibitor activities in plant extracts have been used, and virtually all workers have expressed inhibitor activities in different ways [1]. With only a few exceptions, it has been realized that it is necessary to pre-incubate  $\alpha$ -amylase and inhibitor for inhibition to take place, rather than simply to incorporate inhibitor directly into a digest containing a mixture of enzyme and substrate. However, for the most part, the duration, pH, temperature and other conditions of pre-incubation appear to have been arbitrarily chosen. Amylase inhibitor activities have in many instances been expressed in units, based on the amount of inhibitor which will inhibit a certain proportion of a fixed amount of  $\alpha$ -amylase. It has not usually been made clear whether

the measured extent of inhibition represents the maximum obtainable with the amount of inhibitor used, or whether it is less than the maximum obtainable with that amount of inhibitor. In the latter case, inhibitor activity is being measured in terms of the rate of  $\alpha$ -amylase inhibition, rather than reflecting the stoichiometry of amylase-inhibitor interaction.

By using purified phaseolamin, we have investigated the conditions of pre-incubation which give a reliable assay of inhibitor activity. A procedure based on measurement of the rate of inhibition of hog pancreatic or human salivary  $\alpha$ -amylase has been developed for the assay. One of the most important factors to be taken into consideration is the pH of pre-incubation; maximum rate of inhibition occurs at a pH (5.5) which differs markedly from the optimum pH (6.9) for  $\alpha$ -amylase action. Pre-incubation should be performed at above ambient temperature; the rate of inhibition of  $\alpha$ -amylase at 25 °C is only about 10% of the rate at 37 °C (Fig. 1). The amount of inhibitor used, and the duration of pre-incubation should be such that not more than 50% inhibition of the  $\alpha$ -amylase takes place during pre-incubation, since only under these conditions is the extent of inhibition proportional to the amount of inhibitor and the duration of pre-incubation.

By using the assay procedure which has been developed, the levels of inhibitor activity in a number of legume extracts were determined (Table 1). All varieties of *Phaseolus vulgaris* tested contained high levels of inhibitor activity, there being little difference between the varieties. Inhibitor activity is absent, or very low, in legumes of other species. In most cases the inhibitory activity towards pancreatic and salivary  $\alpha$ -amylases are closely similar, so that either of these enzymes is equally satisfactory for measurement of inhibitor activity. Jaffé and co-workers [8] have reported much greater differences between the amounts of inhibitor activity in different varieties of *Phaseolus vulgaris*. In their experiments, however, the assay conditions were not controlled in the manner we have shown to be necessary.

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## Zusammenfassung

Prüfung der  $\alpha$ -Amylaseinhibitor-Aktivität in Hülsenfrüchten. Es werden die Faktoren diskutiert, welche die Aktivität des  $\alpha$ -Amylaseinhibitors Phaseolamin aus Nierenbohnen, *Phaseolus vulgaris*, beeinflussen; dazu wird das Verfahren zur Prüfung der Inhibitoraktivität beschrieben. Die Größe der  $\alpha$ -Amylaseinhibitor-Aktivität in einer Anzahl von Hülsenfrüchten wird mitgeteilt.

## Résumé

Essai de l'activité inhibitrice vis-à-vis de l' $\alpha$ -amylase dans les légumineuses. Les facteurs qui influent sur l'activité de phaseolamine, un inhibiteur de l' $\alpha$ -amylase du haricot, *Phaseolus vulgaris*, sont discutés, et un mode opératoire pour un test de l'activité inhibitrice est décrit. Les taux de l'activité inhibitrice de l' $\alpha$ -amylase d'un certain nombre de légumineuses sont indiqués.