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AMYLASE INHIBITORS OF *Actiniae* OF THE CARIBBEAN SEA

AMYLASE INHIBITOR OF PROTEIN NATURE FROM *Stoichactis helianthus*

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Natural amylase inhibitors have been found in actiniae of the Caribbean Sea. From the actinia *S. helianthus* an inhibitor has been isolated by gel filtration on Sephadex G-50 and ion-exchange chromatography on CM-cellulose that is highly specific in relation to the amylases of marine mollusks and is inactive in relation of the amylases from other sources.

Amylase inhibitors of protein nature have been detected in wheat and barley [1] and in potato tubers [2], and also in the bacterium *Streptomyces griseosporeus* YM-15 [3, 4]. The amylase inhibitors from wheat have been studied most fully [1, 5-9].

In a systematic search for carbohydrases in marine invertebrates [10] it has been found that extracts of some species of *Actinia* lack the amylase and β-1,3-glucanase activity that is widespread in the animal kingdom. It must be assumed that these sources contain an inhibitor of carbohydrases.

We present the result of a study of the protein amylase inhibitors which we have detected in actiniae of the Caribbean Sea.

The inhibiting capacity of an extract of one of the actinia — *Stoichactis helianthus* — was tested on 13 carbohydrases (six β-1,3-glucanases and seven amylases) from various sources (see Table 1). It can be seen from Table 1 that the amylase activities of the crystalline styles of the mollusks *Chlamys abbidus* [11], *Spisula sachalinensis* [12], and *Patinopecten* sp. [12] are effectively inhibited by an extract of *S. helianthus* (1-5 μg/ml). The α-amylase of human saliva was completely inhibited at concentrations of the extract of about 1 mg/ml. The remaining amylases, and also β-1,3-glucanases from various sources were not inhibited in the range of concentrations of the extract tested. It is possible that the inhibitor contained in *S. helianthus* is specific in relation to the amylases of marine mollusks.

These facts do not contradict the behavior of known protein inhibitors. Thus, the specificity of the inhibitors from wheat were tested on 66 amylases from various sources [6]. The amylases of plants, birds, and mammals were far less subject to their action than the amylases of marine origin and those from insects. The inhibitors from the bacterium *S. griseosporeus* YM-25 acted only on α-amylases of animal origin [3].

It can be seen from Table 2 that the inhibiting capacity of an extract of *S. helianthus* is 2-3 orders of magnitude higher than for extracts of other species of actiniae with respect to the amylase of *Ch. abbidus*. They did not act on the β-1,3-glucanase of *Ch. abbidus*.

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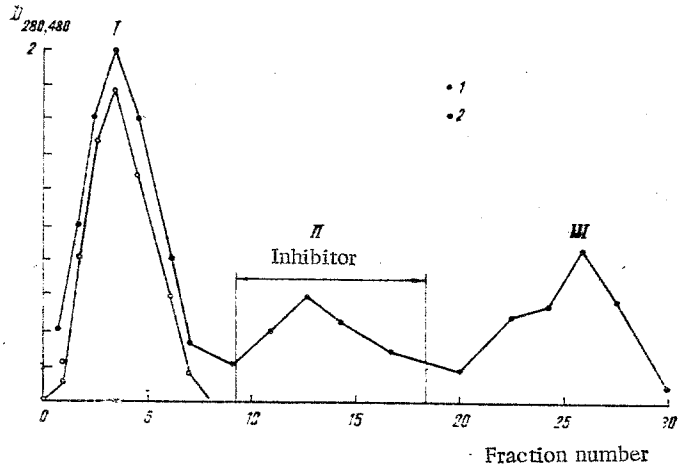

Fig. 1. Gel filtration of a *S. helianthus* extract on Sephadex G-50: 1) hydrocarbon content; 2) protein, D_{280}.

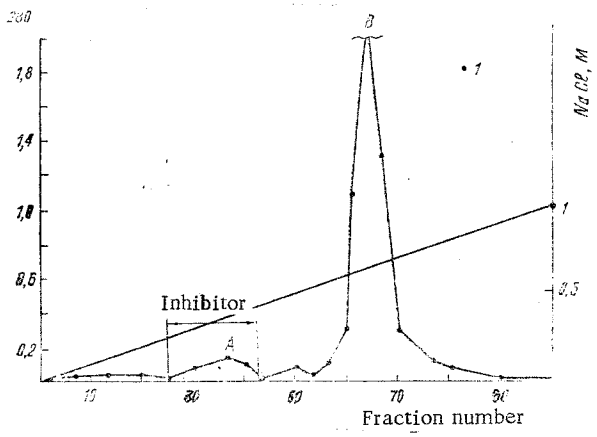

Fig. 2. Chromatography on CM-cellulose: 1) protein, D_{280}.

For a further study of the properties of the *S. helianthus* inhibitor and for developing a scheme of purification, we selected the amylase of *Ch. abbidus*, which most effectively undergoes the action of this inhibitor.

As the first stage of purifying the inhibitor we used gel filtration of the total extract of the actinia on Sephadex G-50, where the protein substances of the extract were separated into three peaks (Fig. 1) [14]. The substances of carbohydrate nature were eluted together with protein peak I, and the inhibiting activity was concentrated in protein peak II.

To separate the substances of peak II we used CM-cellulose (Fig. 2). The amylase-inhibiting activity was found in peak A. The fractions containing the inhibitor were combined and rechromatographed.

A highly purified preparation of the substance possessing the inhibiting capacity had a typical protein spectrum. The optical density at 280 nm (D_{280}) of a 0.1% solution of the inhibitor was 1.1. The molecular weight of the protein determined by gel filtration [15] was about 7500 (Fig. 3).

The protein was distinguished by high thermal stability: heating it at 100°C for three hours did not affect its inhibiting capacity. It is interesting that the known protein amylase inhibitors also have low molecular weights and possess a high thermal stability [2-4, 9], thus, for example, the molecular weight of each of the two inhibitors from *S. griseosporeus* YM-25 is 8500 [3].

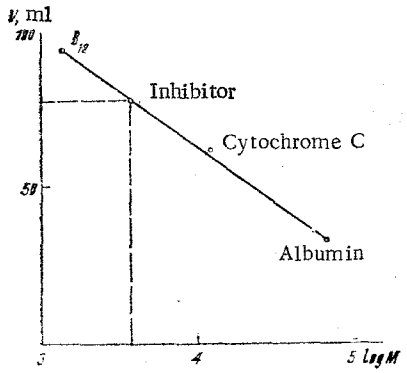

Fig. 3

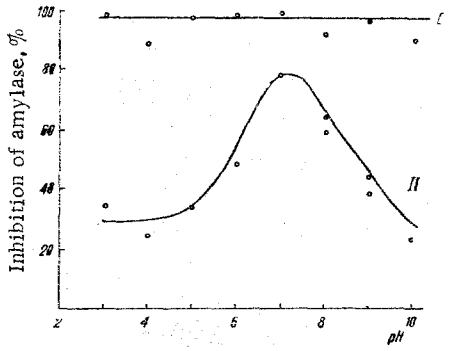

Fig. 4

Fig. 3. Determination of the molecular weight of the *S. helianthus* inhibitor.

Fig. 4. Influence of the pH on the stability of the *S. helianthus* inhibitor at concentrations of the inhibitor of 20 μg/ml (I) and 1 μg/ml (II).

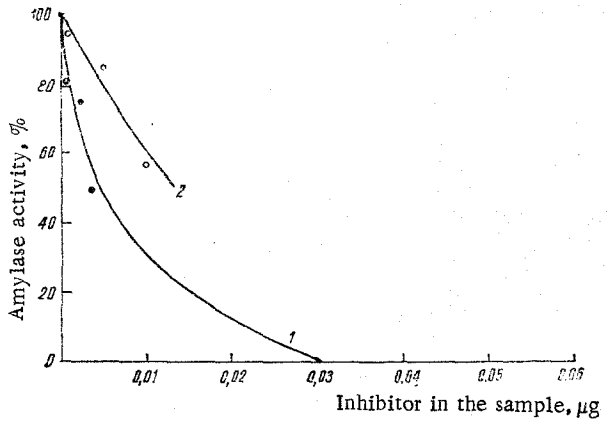

Fig. 5. Action of the *S. helianthus* inhibitor on the amylases of *Ch. abbidus* (1) and *S. sachalinensis* (2) (0.5 of a unit of the enzyme in the sample).

The protein nature of the inhibitor is also shown by the fact that under the action of trypsin it lost 30% of its activity.

We studied the action of a known protein modifier — N-bromosuccinimide — on the inhibitor. The activity of the inhibitor after reaction under conditions typical for the modification of tryptophan residues [16] fell by only 20%.

In relatively low concentrations (20 μg/ml), the inhibitor was fairly resistant to a change in the pH of the medium (Fig. 4, I). The dilution of its solutions raised the sensitivity of the inhibitor to such treatment (Fig. 4, II). It may be considered to be most stable in the pH range of 6-9.

It was of interest to compare the action of various concentrations of purified inhibitor on the amylases of *Ch. abbidus* and *S. sachalinensis* (Fig. 5). It can be seen from Fig. 5 that 50% inhibition of 0.5 of a unit of the amylase of *Ch. abbidus* was achieved at a concentration of inhibitor of 0.004 μg in the sample. A 50% inhibition of 0.5 of a unit from *S. sachalinensis* was observed at a concentration of inhibitor in the sample three times greater (0.012 μg). A similar dependence of the inactivation of amylases from various sources on the concentration of inhibitor has been reported for one of the inhibitors from wheat [7].

The bond between the enzyme and the inhibitor is fairly stable. Gel filtration of the enzyme–inhibitor complex in which there was complete suppression of amylase activity on a

TABLE 1. Influence of an Extract of *S. helianthus* on β-1,3-Glucanase

Concentration of the extract, μg/ml	β-1-3 Glucanase activity,%					
	S. sachalinensis (L III, endo) [12]	S. sachalinensis (L IV, endo) [12]	Ch. abbidus (L 0, endo) [11]	Patinopecten sp. [12]	Eulota maakii (L II, exo) [13]	β-amylase (exo) preparation
1000	100	96	98	100	85	100
500	98	98	100	96		
100	100	100	95	98		
50	97	100	97	100		
10	99	98	100	100		
5	100	100	96	98		
1						
0,2						
0	100	100	100	100	100	100

TABLE 2. Action of Actinia Extracts on the Amylase of *Ch. abbidus*

Concentration of extract, μg/ml	Activity of enzyme,%				
	S. helianthus	Rodactis sancti-thomae	Condylactis gigantea	Buvodosoma cavernata	Phylactis conguilega
1000	0	0	50	0	0
500	0	85	100	20	45
100	0	100	98	100	99
5	0	98	99	98	98
1	100				
0	100	100	100	100	100

column of Sephadix G-75 with increased ionic strength did not lead to the restoration of the amylase activity.

The formation of stable enzyme–inhibitor complexes has also been shown for bacterial protein inhibitors [4] and those from potato tubers [2].

EXPERIMENTAL

Preparation of Total Extracts of the Actininae. The animals were collected from the coast of Cuba near Havana. The actiniae were completely homogenized in 0.9% sodium chloride solution (ratio by weight 1:3). The homogenate was centrifuged at 8000 rpm for 20 min, and the supernatant was freeze-dried. The combined extracts so obtained were stored at 4°C.

Standard Procedure for the Inhibition of Carbohydrases. A mixture containing from 0.2 μg to 100 μg of actinia extract (or from 0.001 μg to 0.05 μg of purified inhibitor) and about 0.5 of a unit of carbohydrase activity in phosphate buffer, pH 6.0 (1 unit = 1 mmole of glucose/h) was kept at 25°C for about 15 min, and solutions of the appropriate substrates were added. The final concentration of the substrate was 1 mg/ml of incubation mixture. The mixtures were incubated at 25°C for 30 min and the activities of the enzymes were recorded by Nelson's method [17]. The inhibiting capacity of the *S. helianthus* extract was tested on amylases of the crystalline styles of *Ch. abbidus* [11], *S. sachalinensis* and *Parinopecten* sp. [12], the amylase from the snail *E. maakii* [13], the amylase of human saliva, Merck pancreatic α-amylase, Merck β-amylase (amylopectin as substrate), the laminarinases of the crystalline styles of *Ch. abbidus* (L 0) [11], *S. sachalinensis* (L III and L IV) and *Patinopecten* sp. [12], the laminarinase from *E. maaki* (L II) [13], and the laminarinase contained in the Merck β-amylase preparation (with the laminarin from *Laminaria cychariodes* as substrate [18]).

The comparison of the inhibiting actions of extracts of various actininae was carried out by a standard method using amylase and the laminarinase from *Ch. abbidus* [11].

Gel Filtration. A solution of 200 mg of freeze-dried *S. helianthus* extract in 5 ml of 0.05 M acetate buffer, pH 4.0, was deposited on a column of sephadex G-50 (3 × 50) equilibrated with the buffer. The rate of elution was 15 ml per hour and the volume of each fraction was 5 ml. The inhibiting capacities of the fractions were detected by a standard method using *Ch. abbidus* amylase [11] and taking 0.01 ml of each fraction for analysis. The protein in the fractions was measured at λ 280 nm, and the carbohydrate content by the phenol–sulfuric acid method [19].

and Amylase Activities

Amylase activity, %						
S. sachalinensis [12]	Ch. abbidus [11]	Patinopecten sp.	Eulota maakii [13]	saliva α-amylase (endo)	pancreatic α-amylase	β-amylase (exo)
0	0	0	100	0	97	99
0	0	0		20	100	98
0	0	0		60		100
0	0	0		70		100
0	0	0		75		98
0	0	0		95		100
0	25	10				
100	98	100				
100	100	100	100	100	100	100

Ion-Exchange Chromatography. Onto a column (1 × 20) containing CM-cellulose (Whatman-32) equilibrated with 0.05 M acetate buffer, pH 4.0, 100 ml of a solution (D_{280} 0.6) obtained after gel filtration and possessing an inhibiting action was run at a rate not exceeding the rate of separation. Elution was performed with a linear gradient of sodium chloride (0-1 M) in 0.05 M acetate buffer, pH 4.0. The total volume of eluate was 300 ml, the rate of elution 10 ml per hour, and the volume of the fractions 3 ml. The fractions containing the inhibitor were combined and rechromatographed.

The UV spectrum of the inhibitor was recorded on a Cary-219 automatic spectrophotometer.

The molecular weight was determined by gel filtration [15] on a column containing Sephadex G-75 (1.5 × 45). The standards used were bovine albumin (mol. wt. 67000), cytochrome C (mol. wt. 12,500), and vitamin B_{12} (mol. wt. 1350).

Action of Proteolytic Enzymes. To 10 µl of a solution of the inhibitor (D_{280} 0.37 units) were added 200 µl of 0.05 M phosphate buffer, pH 8.0, and 10 µl of a solution of pronase or trypsin (100 µg/ml). The samples were incubated at 37° for two days. The reaction was stopped by boiling for 10 min. All operations were carried out in parallel with a control aliquot of the inhibitor. The inhibitor activity was determined by a standard method using *Ch. abbidus* amylase.

The pH stability of the amylase inhibitor from *S. helianthus* was determined in acetate-phosphate-borate buffers (0.035 M, with respect to each component) using a partially purified preparation of the inhibitor (after gel filtration on G-50). The inhibitor was incubated at various pH values for 48 h, and then all the solutions were brought to pH 6.0 with the aid of 0.2 M phosphate buffer and their inhibiting capacities on the amylase from *Ch. abbidus* were tested in accordance with the standard procedure. The final concentrations of partially purified inhibitor preparation in the incubation mixture were 1 µg and 20 µg/ml.

Action of N-Bromosuccinimide (NBS). The gel-filtration purified preparation of amylase inhibitor (4 ml, D_{280} 0.95) was modified with N-bromosuccinimide by the method of Spende and Witkop [16]. The excess of NBS was separated off by dialysis against phosphate buffer, pH 6.0. The same operations with the exceptions of the modification by NBS were carried out in parallel with another portion of the inhibitor (4 ml, D_{280} 0.95). The reactions of the modified and native inhibitors with the *Ch. abbidus* amylase were performed by the standard procedure.

Determination of the Type of Inhibition. Gel filtration of the inhibitor (0.1 ml), of the amylase from *Ch. abbidus* (0.1 ml), and of the enzyme-inhibitor complex (0.15 ml) was performed on a column (1 × 20) of Sephadex G-75 in 0.05 M phosphate buffer and a 0.3 M solution of sodium chloride (pH 6.0) at the rate of 8 ml/h. Fractions of 1 ml were collected and were analyzed for the presence of amylase and inhibitor. The amylase activity was determined by adding 0.1 ml of amylopectin solution (2 mg/ml) to 0.1 ml of each fraction. After the mixture had been incubated for 1 h, the reducing sugars were recorded by Nelson's method [17]. The inhibiting capacities of the fractions were tested by adding 0.1 ml of the *Ch. abbidus* amylase to 0.2 ml of each fraction and then proceeding by the standard method.

SUMMARY

1. Natural amylase inhibitors have been detected in actiniae of the Caribbean Sea.

2. An inhibitor highly specific in relation to the amylases of marine molluscs has been isolated from *Stoichactis helianthus* and has been characterized.

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IMPROVED METHODS OF OBTAINING N^{im}-TRITYL-SUBSTITUTED HISTIDINE DERIVATIVES

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Two variants are proposed for the synthesis of N^{α}-Boc-N^{im}-tritylhistidiine. The first variant starts from N^{α},N^{im}-di-Boc-histidine, from which the N^{im}-Boc group is removed with hydrazine hydrate. The N^{α}-Boc-histidine formed is esterified with chlorotrimethylsilane, tritylated in the imidazole group, and, after the elimination of the trimethylsilyl protection from the carboxyl group, N^{α}-Boc-N^{im}-tritylglycine is obtained with a yield of 80%. The second variant starts from N^{α},N^{im}-ditritylhistidine, which, by treatment with hydrochloric acid in acetone and then with dilute ammonia, is converted into N^{im}-tritylhistidine. From this, by acylation with di-tert-butyl pyrocarbonate, N^{α}-Boc-N^{im}-tritylhistidine is obtained with a yield of 91%. The acylation of N^{im}-tritylhistidine with other alkoxycarbonylating reagents leads to N^{α}-tert-amyl-, N^{α}-benzyl-, and N^{α}-4-methoxybenzyloxycarbonyl derivatives of N^{im}-tritylhistidine.

The triphenylmethyl (trityl) group is not one of the amino-protective groupings that is most widely used in peptide chemistry [1]. The only exception is formed by trityl-substituted histidine derivatives, which have been studied since the fifties [2-5] and have been used in peptide synthesis even in recent years. It is known from various publications [2-4, 6] that the condensation of N^{α},N^{im}-ditritylhistidine with amino acid esters with the aid of dicyclohexylcarbodiimide leads to the formation of dipeptides with yields of 80-90%. Ditri-

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