30.2% mp 100-102°C, R_f 0.86, and also 3-methylbenzoxazolinethione, yield 76%, mp 126-127°C; according to the literature [1]: mp 127-129°C.

SUMMARY

1. A method has been developed for obtaining 3-vinylbenzoxazolinones and 3-vinylbenzoxazolinethione.

2. It has been shown that by this method it is also possible to obtain 3-methylbenzoxazolinethione in good yield (76%).

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ISOLATION AND PURIFICATION OF BIOPOLYMERS BY AFFINITY CHROMATOGRAPHY. VI.* PREPARATION AND PROPERTIES OF AN AFFINITY ADSORBENT WITH A POLYSACCHARIDE SPACER FOR THE PURIFICATION OF PROTEOLYTIC ENZYMES

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A new affinity sorbent has been synthesized — soybean trypsin inhibitor (STI)-amylopectin-hydrazidosuccinyl-Sepharose — and its properties have been studied in comparison with those of an analogous adsorbent without the spacer STI-Sepharose. The STI-amylopectin-hydrazidosuccinyl-Sepharose adsorbent has been used for the purification of trypsin from porcine pancreas and of callicrein from human blood plasma.

Affinity chromatography (AFC) on immobilized soybean trypsin inhibitor (STI) is widely used to purify proteolytic enzymes [2]. Thus, highly purified preparations of trypsin [3, 4] and of callicrein from human blood plasma [5, 6] has been obtained by AFC on STI-Sepharose.

As a rule, the STI is attached to the Sepharose by the cyanogen bromide method [4, 7]. The direct attachment of a high-molecular-weight ligand to a solid support can create substantial steric hindrance for the subsequent interaction of the immobilized ligand with the active center of the enzyme to be isolated which, in the final account, will lead to a decrease in the capacity of the affinity adsorbent. In this connection the use of a spacer separating the ligand from the surface of the solid support could lead to an improvement in the quality of the adsorbent. One of the recent examples of the immobilization of STI on Sepharose through a spacer is the work of Karube et al. [8], who used the activation of Sepharose containing amino groups with the aid of glutaraldehyde and the subsequent attachment of the STI.

*For Communication V, see [1].

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We have recently [9] proposed the use for the purification of biopolymers of various classes of AFC based on biospecific adsorbents with polysaccharide spacers. The principle of polysaccharide spacers was realized previously [10] in the isolation of poly(A)-mRNA on poly(U)-containing adsorbents with dextran or glucogen spacers. These adsorbents were characterized by a high specific content of the ligand and a higher poly(A)-binding capacity than the corresponding adsorbent without a spacer — poly(U)-Sepharose. It appeared of interest to see whether affinity adsorbents with polysaccharide spacers containing two ligands would possess similar advantages, in particular, in the purification of biopolymers of protein nature.

In the present paper we consider the synthesis of properties of a new affinity adsorbent with an amidopectin spacer for the purification of proteolytic enzymes of the serine class — "STI-amylopectin-hydrazidosuccinyl-Sepharose" (adsorbent A). Some properties of this adsorbent are compared with the properties of the analogous adsorbent without the spacer — "STI-Sepharose" (adsorbent B) — in the affinity chromatography of trypsin from porcine pancreas and of callicrein from human blood plasma.

To synthesize adsorbent A, hydrazidosuccinyl-Sepharose [10] was treated with periodate-oxidized amylopectin at pH 4.8. Amylopectin was oxidized under conditions to modify approximately 5% of the anhydroglucose residue. After the addition of the amylopectin to the Sepharose, the residual aldehyde groups in the adsorbent were reduced with sodium tetrahydroborate, the polysaccharide spacer was activated with BrCN, and the STI was added under the usual conditions [9]. Adsorbent B was obtained by the standard method — by the addition of STI to Sepharose 4B after its activation with BrCN. The content of STI in the adsorbents obtained was determined by the differential method from the difference in the amount of ligand in the initial solution of STI and in the wash-waters after addition. According to the results obtained, the amounts of STI in adsorbents A and B ranged between 7 and 9 mg/ml of gel.

The antitryptic activity of the immobilized STI was determined from the capacity of a suspension of the adsorbent for inhibiting the cleavage of the ethyl ester of N-benzoylarginine (Bz-Arg-OEt) by trypsin. The spectrophotometric determination of the degree of cleavage of the substrate at 253 nm was carried out directly in a suspension of the gel stabilized by the addition of a 0.1% solution of agarose [11]. The use of a 0.1% solution of agarose ensured the reliable determination of absorption in the suspension of the gel. The results obtained indicated that the specific antitryptic activity of STI immobilized through an amylopectin spacer was almost twice that of the activity of the STI directly attached to Sepharose (Table 1). The use of the polysaccharide spacer thus permitted the retention of 20% of the activity of the immobolized STI in relation to the activity of the inhibitor in solution. Apparently the polysaccharide spacer, by ensuring a considerable distance of the inhibitor from the surface of the solid support, leads to a retention of the conformation of the protein closer to the native conformation than when the STI is attached directly to the Sepharose.

The capacity of the adsorbents A and B with respect to trypsin and callicrein, enzymes exhibiting practically the same affinity for the native soybean inhibitor (inhibition constants of the order 10^{-10} M [10, 12], was determined. The sorption of the proteinases was carried out in 0.1 M sodium phosphate buffer solution (pH 6.2) in the presence of 0.2 M of sodium chloride with deliberate saturation of the adsorbent by the enzyme. After the elimination of the ballast proteins by washing the column with the equilibrating buffer solution, the elution of the bound proteinase was achieved with 0.01 M hydrochloric acid containing 1 M sodium chloride. It was found that the capacity of the adsorbent with the polysaccharide spacer in relation to both enzymes was about three times greater than the capacity of the STI-Sepharose (Table 1). It may be assumed that the observed increase of the capacity of adsorbent A is explained by the greater accessibility of the reactive center of the immobilized STI for the active center of the enzyme.

The results on the purification of a commercial preparation of trypsin from porcine pancreas and the partial purification of a preparation of callicrein from blood plasma on adsorbents A and B under the conditions described above are given in Table 2. The yields, specific activities, and degrees of purification of these enzymes on both adsorbents were practically the same. A substantial advantage of the purification of proteinases on STI-amylopectin-Sepharose as compared with the adsorbent without the spacer is the production of a final enzyme preparation in a far smaller volume of eluting buffer solution. A 2- to 2.5-fold increase in preparations of trypsin purified by AFC on adsorbent A as compared with AFC on STI-Sepharose is extremely important for subsequent work with these enzymes.

TABLE 1. Properties of Affinity Adsorbents Containing STI

Adsorbent	Antitryptic activity, IU/mg of immobilized STI	Capacity with respect to trypsin		Capacity with respect to callicrein	
		U/ml of gel	mg/ml of gel	U/ml of gel	mg/ml of gel
A	4,4	100	2,5	56	2,8
B	2,4	30	0,75	20	1

TABLE 2. Purification of Trypsin and Callicrein on Adsorbents A and B

Adsorbent	Fraction	Trypsin					Callicrein				
		protein, mg	protein, mg/ml	sp. act., U/mg	yield %	degree of purification	protein mg	protein mg/ml	sp. act., U/mg	yield %	degree of purification
A	Initial preparation	20	0,095	18	—	—	836	1,04	0,25	—	—
	Eluate I (equilibrating buffer solution	11	0,157	3,3	9	—	800	5,7	0,00	—	—
	Eluate II (0,01 M HCl+1 M NaCl)	8,5	0,485	38	89	2,1	10	0,57	20	96	80
B	Initial preparation	6	0,095	18	—	—	420	1,04	0,25	—	—
	Eluate I (equilibrating buffer solution)	3,5	0,05	12	11	—	410	2,9	0,02	14	—
	Eluate II (0,01 M HCl+1 M NaCl)	2,4	0,137	38	85	2,1	4,5	0,26	20	85	80

It must be mentioned that after the desorption of the proteinase on the STI-amylopectin-Sepharose and the regeneration of the adsorbent the inhibitory activity of the immobilized STI and the capacity of the adsorbent were practically unchanged.

Thus, the introduction of a polysaccharide spacer between the STI and the surface of the solid support improves the properties of the affinity adsorbent and raises the efficiency of the biospecific purification of proteolytic enzymes on it.

EXPERIMENTAL

We used Sepharose 4B (Sweden), soybean trypsin inhibitor from Hungary, porcine pancreatic trypsin from Czechoslovakia, N-benzoylarginine ethyl ester from the United Kingdom and the other reagents of high-purity grade from Soyuzreaktiv (USSR).

The activation of the Sepharose with BrCN was carried out by a known method [9]. The concentration of protein was determined from the absorption in the UV region at 280 nm.

STI-amylopectin-hydrazidosuccinyl-Sepharose (Adsorbent A). A suspension of 10 ml of BrCN-activated Sepharose 4B in a solution of 960 mg of succinic acid hydrazide in 10 ml of water was stirred at 4°C for 16 h and was then washed with 100 ml of water and with 50 ml of 0.1 M sodium acetate buffer solution (pH 4.8). The hydrazidosuccinyl-Sepharose obtained was suspended in a solution of 972 mg of periodate-oxidized amylpectin [9] in 10 ml of 0.1 M sodium acetate buffer solution (pH 4.8). The mixture was stirred at 4°C for 16 h and was then washed with 100 ml of 2 M NaCl and with 100 ml of water and was stirred in 100 ml of 0.1 M $NaHCO_3$ with 200 mg of $NaBH_4$ at 4°C for 3 h.

The amylopectin-hydrazidosuccinyl-Sepharose was washed with water (50 ml) and was activated in 10 ml of 5 M potassium phosphate buffer solution, pH 11.9, with the aid of 1.5 g of BrCN. The activated gel was washed with cold water and with 0.1 M $NaHCO_3$ (50 ml each) and was rapidly mixed with a solution of 100 mg of STI in 1 ml of 0.1 M $NaHCO_3$ containing 0.5 M NaCl. The suspension was stirred at 4°C for 16 h, and then the adsorbent was washed with 0.1 M $NaHCO_3$ containing 0.5 M NaCl until the wash-waters no longer adsorbed at 280 nm, and it was then washed alternatively with 50-ml portions of 0.1 M sodium borate buffer solution containing 1 M NaCl (pH 4) (three washing cycles) and with water, and the residual active groups in the adsorbent were blocked with 1 M ethanolamine (pH 9). Adsorbent A was stored in aqueous suspension in the presence of 0.2% of sodium azide.

STI-Sepharose (adsorbent B) was obtained by the method of Porath et al. [7].

Determination of the Antitryptic Activity of the Immobilized STI. Aliquots of adsorbents A and B (0.01-0.10 ml) were brought to a volume of 1.4 ml with Tris-HCl buffer solution, pH 8.0, and then 0.1 ml of a solution of 10 μg of trypsin in 2.5 mM HCl was added. The mixture was carefully stirred and the kinetics of the hydrolysis of the substrates were recorded from the absorption at 283 nm. In a control sample, in place of the aliquot of adsorption at 253 nm. In a control sample, in place of the aliquot of the adsorbent the corresponding amount of unsubstituted Sepharose 4B was added.

The activity of the adsorbents was expressed in inhibitor units per 1 mg of immobilized STI. 1 IU is the nominal inhibitor unit corresponding to the amount of inhibitor inhibiting the cleavage of 1 μmole of substrate in 1 min under the conditions given above.

The determination of the esterase activities of the trypsin and callicrein was carried out spectrophotometrically at 253 nm at 25°C in 0.05 M Tris-HCl buffer solution using BzArgOEt as substrate. The activity of the proteinases was expressed in units per mg of enzyme. 1 U is the nominal unit corresponding to the amount of enzyme capable of cleaving 1 μmole of substrate in 1 min under the standard conditions.

Affinity Chromatography of Callicrein and Trypsin. Solutions of a partially purified preparation of callicrein from human blood plasma* (Table 2) were deposited at the rate of 12 ml/h on a column (4 × 0.8 cm) containing absorbent A or B equilibrated with 0.1 M sodium phosphate buffer solution containing 0.2 M NaCl (pH 6.2). The columns were washed with the equilibrating buffer solution, which eluted the ballast proteins (eluate I, see Table 2). The complex of callicrein with STI was decomposed with 0.01 N HCl containing 1 M NaCl. Elution was carried out at the rate of 48 ml/h, 2.5-ml fractions being collected with ice cooling. Each fraction was rapidly brought to pH 8.0 with 3.5 N NaOH, and its esterase activity was determined.

The AFC of trypsin was performed analogously, and the quantitative results of this process are given in Table 2.

SUMMARY

1. A new affinity adsorbent with a polysaccharide spacer, STI-amylopectin-hydrazidosuccinyl-Sepharose, has been synthesized and its properties have been studied in comparison with those of the analogous adsorbent without the spacer — ATI-Sepharose.

2. Trypsin from porcine pancreas and callicrein from human blood plasma has been purified on the new adsorbent.

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*The partial purification of callicrein from human blood plasma using chromatography on DEAE-Sephadex A-50 and SP-Sephadex C-50 will be described in a separate communication.