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Linear Copolymerization of Unsaturated Albumin Derivatives with Acrylamide

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Summary

The radical copolymerization of macromonomer - human serum albumin N-acryloyl derivative containing one unsaturated bond with acrylamide has been investigated. It has been found that the water-soluble copolymer of molecular mass about 10^6, containing 4-5 chemically bound molecules of albumin is formed.

Introduction

The method of immobilization of physiologically active species which consists of copolymerization of their unsaturated derivatives (1,2) gives a wide possibility for synthesis of new systems for biochemistry and medicine. The usage of copolymerization technics includes introduction of reactive double bond into protein or enzyme molecule. This stage as well as polymerization itself should be carried out in mild conditions to prevent the possible denaturation of initial substance.

It has been found earlier that it is possible to obtain N-acryloyl and N-methacryloyl derivatives of human serum albumin (HSA), in which acryloyl fragments are bound to asparagine and lyzin fragments (3), and during this processes albumin is not denaturated retaining its capability to bind various organic compounds.

The aim of this paper is the synthesis of monoacylated HSA and study of its copolymerization with acrylamide to obtain water-soluble copolymers.

Experiment

Synthesis of N-acryloyl HSA derivative (N-AHSA)

0.01 ml of isopropanol was added to 1 g of HSA($1.4\ 10^{-5}$M), dissolved in 10 ml 5% bicarbonate buffer at pH 8 and 3-5°, and at vigorous stirring 0.1 ml acryloyl chloride solution (1 ml in 30 ml of dioxane, $2.1\ 10^{-5}$M) is added. Reaction mixture is maintained at 3-5° with vigorous stirring for 2 hours. After the reaction was completed N-AHSA is separated from low molecular products by gel-filtration on Sephadex G-10 and liophilized. Yield 90%.

Double bond content measurement in N-AHSA was carried out according to (4) by titration of free amino groups N-AHSA with trinitrobenzenesulfonic acid (TNBS). Absorption of N-

AHSA-TNBS complex was registrated at 420 nm. The number of double bonds was determined as difference 60-n, where n - number of titratable aminogroups in N-AHSA molecule, 60 - number of titratable amino groups in HSA molecule.

Copolymerization of N-AHSA with acrylamide

0.01 ml of N,N,N,N-tetramethyl ethylendiamine was added to 1 ml of water containing 0.1 g (1.4×10^{-6}M) N-AHSA and 0.03 g (4.2×10^{-4}M) of acrylamide (AAm), then the mixture was evacuated and 0.1 ml $(NH_4)_2S_2O_8$ (0.1 g/ml) added. The reaction mixture was kept at 35° for various polymerization times - 15, 30, 120 and 300 min. The isolation of water-soluble complex N-AHSA-AAm from reaction mixture was carried out by gel-filtration on Sepharose 4B (LKB, Sweden, gel volume 60 ml, flow rate - 0.8 ml/min, dead volume - 26 ml, elution time - 90 min, eluent - phosphate buffer). The copolymer is eluated from the column in a dead volume then HSA and low molecular products in a volume 47-56 ml are eluated. Absorbance was registered at 280 and 220 nm. Yield of copolymer ~30%.

Separation of water-soluble copolymer N-AHSA-AAm and AAm homopolymer was achieved by affinity chromatography on Phenyl-Sepharose (LKB, Sweden, 10×300 mm, gel volume - 20 ml, flow rate - 0.9 ml/min, eluent - phosphate buffer (40 ml) and 70% ethanol solution in water (60 ml), elution time - 2 hours). Fraction of 50-55 ml was collected and both HSA and copolymer content were determined.

In ultracentrifugation experiments a Beckman's centrifuge (USA) (rotation rate - 56000 rpm) has been used. The sedimentation constant S_o was calculated accordingly (5). Determined S_o value for N-AHSA-AAm was 6.67×10^{-13}sec^{-1}.

Intrinsic viscosity $[\eta]$ was determined in accordance with (6) in phosphate buffer solution at 22°. $[\eta]$ of copolymer was 0.699 dl/g.

UV-spectra were registered with Pye Unicam SP8-100.

Results and discussion

In accordance with previously published results (3) the reaction of HSA and acryloyl chloride at nearly equimolar ratio proceeds with the formation of unsaturated derivative of albumin when the acryloyl group is attached to the terminal asparagine. As a result the isoelectric point of HSA is shifted when one double bond is introduced in HSA molecule as it follows from absorption spectra of N-AHSA-TNBS and HSA-TNBS. It could be expected that synthesized macromonomer of albumin can participate in the copolymerization reaction with some monomers to give water-soluble copolymers.

As seen from fig.1, the result of copolymerization is a product of high molar mass which contains albumin and it appears in the dead volume of column. When the initial reaction mixture contains AAm and nonmodified HSA (the control experiment, curve 2) the adsorbance corresponding to high molar mass fraction is not detected and peak, corresponding to AAm (in comparison with curve 3) disappears. This means that polymerization of AAm takes place but polymer formed does not contain a protein.

The yield of copolymerization product depends on the con-

centration of initiator; conversion of N-AHSA is increased slower then AAm conversion (fig.2).

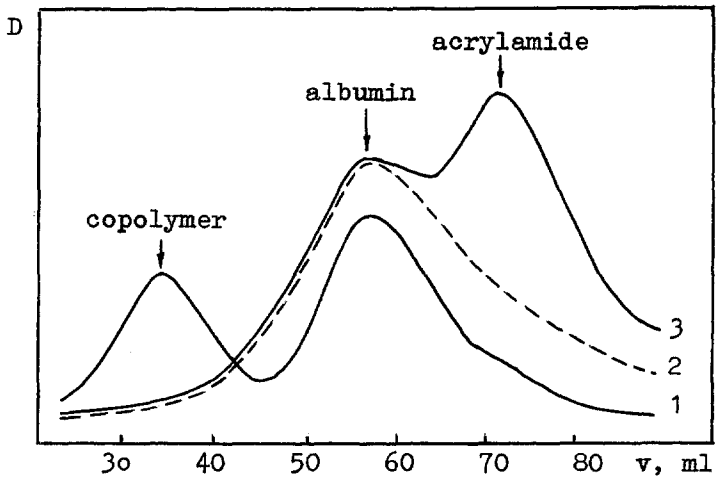


Fig.1. The dependence of eluent absorption at 275 nm on eluent volume passed through the Sepharose 4B. [N-AHSA] = 1.45×10^{-6}M, [AAm] = 4.2×10^{-4}M, polymerization time - 30 min. 1 - reaction mixture after copolymerization, 2 - AAm polymerization in the presence of HSA, 3 - initial reaction mixture

There is a difference between the N-AHSA conversion and fraction of N-AHSA which is entering into the water-soluble copolymer.

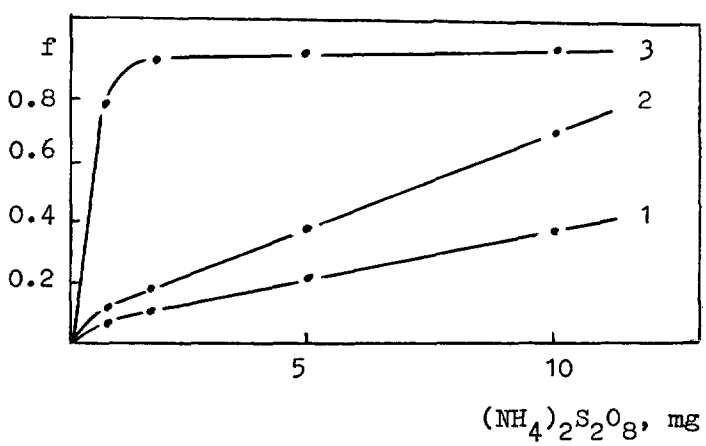


Fig.2. Conversion of monomers versus concentration of initiator: 1 - N-AHSA in water-soluble copolymer, 2 - N-AHSA, 3 - AAm

The latter is related with the formation of cross-linked copolymer of AAm and N-AHSA apparently due to the chain transfer reaction with polyacrylamide chains. The same gel formation is observed in the case of AAm polymerization without N-AHSA.

It should be noted that the N-AHSA homopolymerization does not take place, but AAm homopolymer is detected in the reaction mixture. As it is seen from fig.3 the copolymer's yield depends on the reaction time and reaches its maximum after 30-40 min.

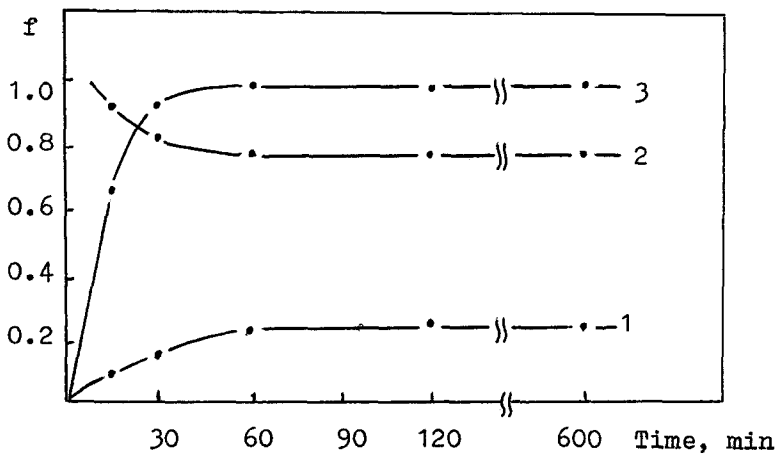


Fig.3. The dependence of water-soluble copolymerization products yield versus time. 1 - f N-AHSA, 2 - (1-f) N-AHSA, 3 - f AAm, where f - degree of conversion

At this time the AAm and N-AHSA conversion are 90-95% and 15-20% respectively. Further increase of the time practically has no influence on the copolymer's yield.

To separate N-AHSA-AAm copolymer and AAm homopolymer affinity chromatography was used (Fig.4).

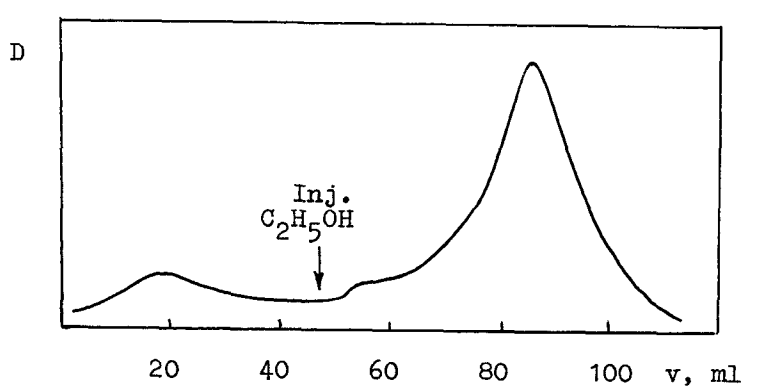


Fig.4. The high-molecular fraction of copolymerization N-AHSA with AAm product chromatogramme on Phenyl-Sepharose. Eluents: phosphate buffer solution, pH 7.4; ethanol 70%; λ = 275 nm

High-molecular fraction, consisting of N-AHSA-AAm copolymer and AAm homopolymer and isolated by gel-filtration was separated on the affinic carrier. The homopolymer appeared with buffer solution in a dead volume and copolymer was eluated further on with 70% ethanol solution. This fact proves the presence of albumin in the copolymer and indicated the albumin ability for affinic binding.

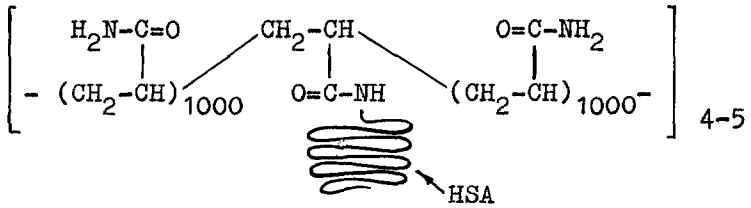
The molecular mass of N-AHSA-AAm copolymer was determined from intrinsic viscosity data and sedimentation constant value obtained after ultracentrifugation experiments using the Flory-Mandelkern-Tsvetkov invariant (5,7). The molecular mass of N-AHSA-AAm copolymer was found to be $\sim 10^6$, as indicated in Table 1.

Table 1. N-AHSA-AAm water-soluble copolymer molecular mass parametrs.

Compound	Sedimentation constant, $S_o \times 10^{13}$	Intrinsic viscosity dl/g	Specific partial volume, v_o	Molecular mass
Human albumin	4.31	0.037	0.734	$6.9\ 10^4$
Poly AAm*)	2.39	0.491	0.658	$5.3\ 10^4$
N-AHSA-AAm copolymer	6.67	0.699	0.880	$0.9\ 10^6$

*) (5)

Weight ratio N-AHSA:AAm in the copolymer was determined from UV-checking of HSA content and gravimetric measurements after lyophilization. On the base of these experiments N-AHSA-AAm copolymer structure may be presented as 4-5 HSA molecules linked with polyAAm chains of molecular mass of 1.5×10^5 each. The schematic structure of this copolymer can be presented as:



The question whether this copolymer is linear or branched needs additional study.

Thus data presented implies the formation of albumin macromonomer as the result of albumin acylation with acryloyl chloride and this macromonomer can take part in the copolymerization with common water- soluble monomers to form a soluble copolymer.

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