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RNA-hydrolyzing activity of human serum albumin and its recombinant analogue

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ABSTRACT

The comparative analysis of RNA-hydrolyzing activity of albumin from human serum and albumin expressed in methylotrophic yeast *Pichia pastoris* has been carried out. The rate of polyribonucleotide phosphodiester bond cleavage in the presence of recombinant albumin has been found to be similar to that of the reaction mediated by the native protein. According to ^{31}P NMR data, RNA hydrolysis follows the mechanism of intermolecular trans-esterification to yield 2′,3′-cyclophosphodiester reaction products that are further slowly hydrolyzed to form nucleoside-3′- and nucleoside-2′-phosphates. Analysis of pH dependence suggests an acid–base mechanism of catalysis. The catalytic activity and substrate specificity of albumin in RNA hydrolysis distinguish it from human ribonucleases.

Human serum albumin (HSA) is a biological macromolecule that has the potential for a wide range of pharmaceutical applications.[1] It is usually isolated by human plasma fractionation, which has the potential risk of albumin contamination with blood-derived pathogens. To overcome the problem of contamination and to find a more convenient and consistent protein source, substituting native albumin with recombinant HSA (rHSA), which is successfully produced by using a methylotrophic yeast, *Pichia pastoris*,[1b,2] has been suggested recently. It was demonstrated that purified rHSA is identical to plasma-derived HSA with no detectable mannan component from *P. pastoris*.[1b] Preclinical and clinical trials have confirmed the safety and efficacy of rHSA preparation for its usage as a plasma expander in critical health conditions related to plasma volume and oncotic pressure.[1b,c] In addition, rHSA has great potential for other medical and pharmaceutically related applications.[1b] However, despite the structural identity between blood-derived and recombinant albumin, their substitutability in terms of functional properties should be further investigated.

A remarkable functional property of albumin is its promiscuous catalytic activity toward a broad range of organic molecules, including esters, amides, phosphates and benzisoxazoles.[1a,3] Earlier, we have reported on the RNA-hydrolyzing activity of albumin.[4] In this study, we further investigated the RNA-hydrolyzing property of albumin and compared the efficacy of RNA cleavage for the protein isolated from human blood with that for recombinant albumin expressed in *P. pastoris*. We also studied the catalytic mechanism of the protein-mediated oligo- and polynucleotides hydrolysis and proved that both albumin preparations demonstrate acid–base catalysis for RNA phosphodiester bond cleavage.

Recombinant albumin was obtained by expression of the protein in the stable strain of methylotrophic yeast *P. pastoris* created recently.[2] The comparative analysis of CD spectra for the recombinant protein and albumin isolated from donor serum revealed the identity of the proteins' secondary structure. The content of α-helices, β-sheets and β-turns was determined to be 58.9% ± 1.9%, 4.5% ± 0.5% and 13.1% ± 1.2%, respectively, which is in accordance with the data obtained by other scientists for native albumin preparations.[5]

To monitor the reaction of HSA and rHSA with RNA, the methods of denaturing PAGE analysis and ^{31}P NMR spectroscopy were employed using pAGGATCUAUAAAUGAC (ON16), 96-nt fragment of RNA HIV-1 (RNA-96), poly(C), poly(U) or poly(A) as substrates. For the analysis of protein-mediated cleavage the substrates were incubated with RNase-free and fatty acids-free HSA or rHSA at

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37 °C for up to 21 days in a solution containing 50 mM Tris–HCl, pH 7.0, 0.2 M KCl, 0.5 mM EDTA.

According to ^{31}P NMR data, RNA hydrolysis follows the mechanism of intermolecular *trans*-esterification to produce 2′,3′-cyclophosphodiester reaction products, which are further slowly hydrolyzed to form nucleoside-3′- and nucleoside-2′-phosphates. Initial ^{31}P NMR spectra showed disappearance of the intact poly(C) resonances (the typical signal for internucleotide phosphate groups at ~0 ppm) with the concomitant appearance of two groups of resonances (signals at ~20.55 ppm and ~20.95 ppm in Fig. 1,

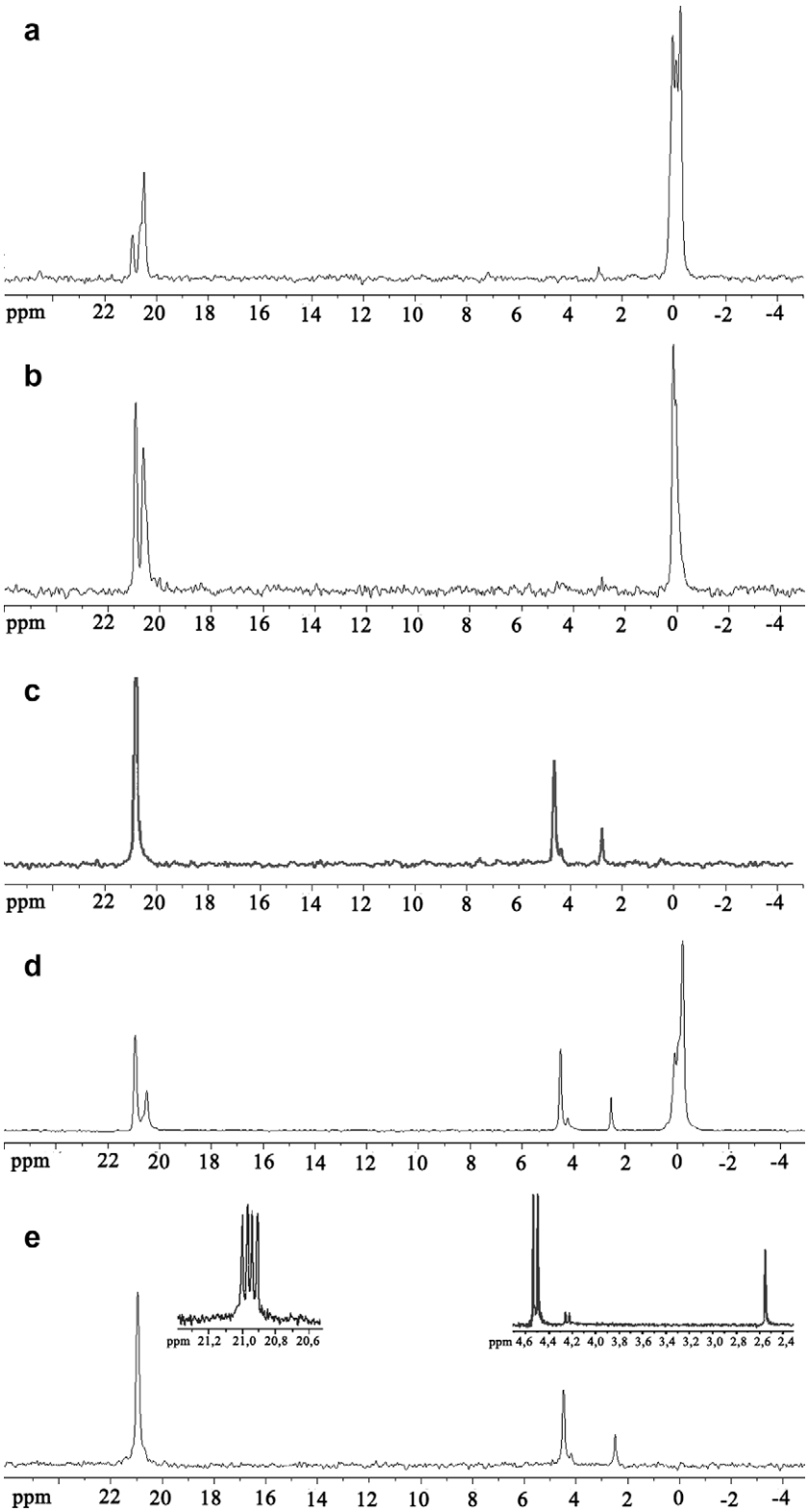

Figure 1. ^{31}P NMR spectra of poly(C)/albumin reaction mixtures. Poly(C) (9.6×10^{-3} M per a phosphate moiety) was incubated with albumin (7×10^{-4} M) at 37 °C for 2 h (***a***), 6 h (***b***) and 72 h (***c***). ^{31}P NMR spectra after the addition of new portion of poly(C) (9.6×10^{-3} M per a phosphate moiety) to the mixture of poly(C) with HSA incubated for 21 days: incubation for 2 h (***d***) and 72 h (***e***).

spectrum ***a***). These signals can be assigned to the oligonucleotides of different length carrying 2′,3′-cyclophosphate group.[6] For longer reaction times (up to 72 h), the cytidine 2′,3′-cyclophosphate peak (vicinal decoupling of the peak at 20.95 ppm with *J* constants of 6.7 Hz and 12 Hz in Fig. 1, spectrum ***e***) was accumulated at the expense of other peaks. This cyclic intermediate was hydrolyzed, presumably by a water molecule, to give 3′-(or 2′-)phosphate (doublets with *J* = 7.6 Hz for the signals at 4.52 ppm and 4.25 ppm, respectively, in Fig. 1, spectra ***c*** and ***e***). The signal at 4.52 ppm was referred to as cytidine 3′-monophosphate according to ^{31}P NMR analysis of the products of poly(C) hydrolysis with RNase A, which is known to preferably convert 2′,3′-cyclophosphate into 3′-monophosphate at the second stage of the hydrolysis reaction.[7]

When a new portion of polycytidylic acid was added to poly(C)/HSA reaction mixture that had been incubated for 21 days (which is equal to the half-life of the protein in the bloodstream[1a]) RNA-hydrolyzing activity of albumin was restored, as shown by the appearance of the downfield signal at 20.64 ppm in the correspondent NMR spectrum (Fig. 1, spectrum ***d***). After 3 days of incubation the signal from phosphodiester groups disappeared, thus proving the polynucleotide cleavage (Fig. 1, spectrum ***e***). Therefore, the obtained data allow us to suggest that HSA is a true catalyst of RNA hydrolysis since it facilitates the reaction but is not consumed in the course of it and is able to participate in multiple rounds of the chemical transformation. It was found that one albumin molecule induced the cleavage of about 13 phosphodiester bonds in poly(C) after 24 h of incubation. The efficiency of the protein as a catalyst of phosphodiester bond hydrolysis depended on the substrate's primary structure. In the case of oligonucleotide ON16 the turnover was higher than for polynucleotide: one albumin molecule resulted in cleavage of 144 phosphodiester bonds after 24 h of incubation.

The bimolecular rate constants for albumin-induced poly(C) hydrolysis, calculated from the time-course decrease of relative integral intensity of the phosphodiester ^{31}P NMR signal, were found to be $(2.7 \pm 0.2) \times 10^2\ M^{-1}h^{-1}$ and $(3.0 \pm 0.1) \times 10^2\ M^{-1}h^{-1}$ for rHSA and HSA, respectively. Similar rate constants speak in favor of functional similarity of two albumin preparations.

The kinetic curves for phosphodiester, 2′,3′-cyclophosphate and 2′- and 3′-monophosphate groups (based on the integral intensities of the correspondent signals observed in NMR spectra of poly(C) incubated with HSA) are demonstrated in Figure 2. It can be seen that the experimental data fit well with the kinetic equations for two subsequent reactions with the compounds carrying 2′,3′-cyclophosphate group being the reaction intermediates. The rate constants calculated from NMR data for both stages are $0.108 \pm 0.001\ h^{-1}$ and $(1.58 \pm 0.08) \times 10^{-3}\ h^{-1}$, respectively. Therefore, HSA is about 70 times more active in the reaction of poly(C) hydrolysis than in the hydrolysis of 2′,3′-cyclophosphodiesters.

The sequence-specificity of RNA cleavage in the presence of native albumin and its recombinant analogue was studied in the experiments with in vitro transcript of 96-nt fragment of HIV-1 RNA containing the primer-binding site (hereafter RNA-96). 5′-[^{32}P]-Labeled RNA-96 was incubated with albumin, and the cleavage products were analyzed by denaturing PAGE (Fig. 3A). According to the products intensity and the time of appearance, the cleavage sites can be divided in two groups: the primary sites, where strong cuts are observed (Fig. 3A, depicted by solid arrows), and secondary sites, where weak cleavage appeared after longer incubation (Fig. 3A, depicted by dashed arrows). Strong cuts are mostly located in the single-stranded regions or regions with unstable secondary structure; weak sites are observed in the double-stranded regions, according to the predicted secondary structure of RNA-96.[8] Both native and recombinant proteins hydrolyzed RNA-96 in a sequence-independent manner. However, the major cleavage occurred within the U–A and C–A sequences (Fig. 3B).

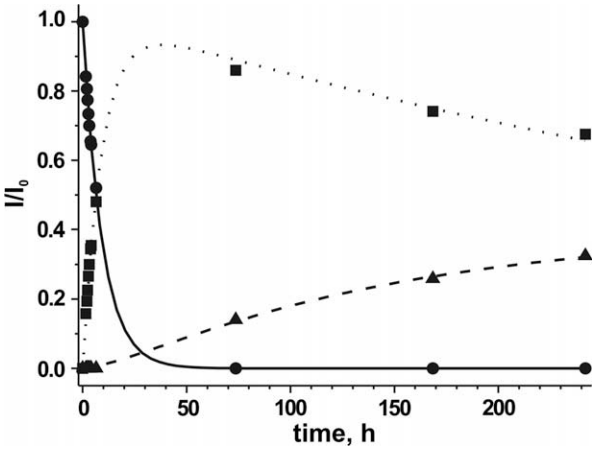

Figure 2. Time-dependence of relative integral intensities for the signals of phosphodiester (●) and 2′,3′-cyclophosphodiester groups (■), as well as cytidin-2′ (3′)-monophosphate (▲). The data are average values of two independent experiments. The error for the integral intensities did not exceed 20%. Curves are the fitting of the experimental data to the kinetic equations for two subsequent reactions with the compounds carrying 2′,3′-cyclophosphate group as intermediates. Poly(C) (9.6×10^{-3} M per a phosphate moiety) was incubated with HSA (7×10^{-4} M) at 37 °C.

It is known that UpA and CpA sequences are major cleavage sites in the reaction catalyzed by RNase A and some other human blood RNases.[9] However, the substrate specificity of human serum albumin in the polynucleotide hydrolysis differs from the specificity of mammal ribonucleases (Table 1). For instance, HSA hydrolyzes polyadenylic acid only about two times less efficiently than poly(U) substrate, whereas the activity of human RNase I toward poly(A) is two orders of magnitude less than toward poly(U).

RNA-hydrolysis can follow several mechanisms. HSA interaction with oligo- or polyribonucleotides can induce the changes in phosphodiester bonds conformation, thus assisting their cleavage ('conformational stress'). In addition, in the complex with a ribonucleic acid the side-chain amino acid groups of the protein can maintain general acid, general base or general acid–base catalysis.

To elucidate a possible mechanism of albumin-mediated catalysis of RNA cleavage and to determine the range of conditions under which the protein exhibits the highest ribonuclease activity, we studied pH profile of ON16 cleavage by HSA.[10] It was observed that the pH profile for HSA is bell shaped: albumin displays the highest activity within the pH interval of 4.9–6.1 with a maximum at pH 5.5 under the conditions used (Fig. 4).

It is interestingly to note that the optimal pH for albumin-catalyzed oligoribonucleotide hydrolysis is acid-shifted, in comparison with the optimal values for RNase A[9b] or human RNase 1,[9d] which have catalytic histidines in their active centers. RNase T1,[11] which has a histidine and a glutamate residue important for the catalysis, has pH optimum close to that observed for albumin. However, the obtained data are insufficient to unambiguously determine the nature of albumin's amino acid residues involved in the catalysis of RNA cleavage, since the ionization constant values for the protein amino acid functional groups highly depend on the protein microenvironment. For example, pK_a for Cys34 of HSA is about 5.0,[1a] whereas the ionization constant for free SH-group is known to be 8.23.[12]

Taken together, the data on specificity and pH dependence of HSA-catalyzed RNA hydrolysis distinguish albumin from the secretory human ribonucleases. Similar efficacy and cleavage pattern for the natural albumin preparation and the recombinant protein can serve as an additional criterion to prove the intrinsic RNA-cleaving properties of albumin. Moreover, the obtained data can contribute

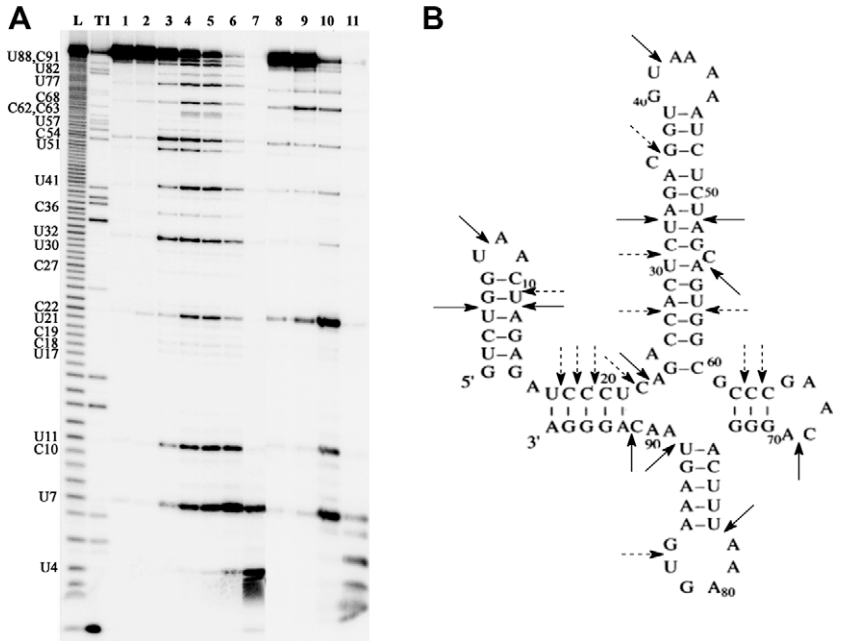

Figure 3. (A) RNA-hydrolyzing activity of HSA (lanes 3–7) and rHSA (lanes 8–11). Autoradiograph of 12% PAAG/8 M urea. Lanes L and T1—imidazole ladder and partial RNA-96 digestion with RNase T1, respectively. Lanes 1, 2—[5′-^{32}P]-RNA-96 incubated in the absence of the albumin for 0 min and 22 h, respectively. Assay conditions: [5′-^{32}P]-RNA-96 was incubated in the presence of albumin (2×10^{-5} M) in 50 mM Tris–HCl buffer, pH 7.0, containing 0.2 M KCl, 10 mM EDTA, 100 μg/ml RNA carrier, at 37 °C for 10 min (lanes 3 and 8), 30 min (lanes 4 and 9), 1 h (lane 5), 2.5 h (lanes 6 and 10), 22 h (lanes 7 and 11). Location of cleavage sites is shown on the left. (B) Secondary structure of RNA-96.[8] Arrows show the sites of cleavage induced by the HSA. The major cleavage sites are depicted by solid arrows. The minor cleavage sites are depicted by dashed arrows.

Table 1
Relative activity of RNase A, human RNases, and HSA for polyribonucleotide hydrolysis[a]

Protein	poly(C)	poly(U)	poly(A)
RNase A[9b]	100%	4.7%	0.005%
Human RNase 1[9d]	100%	3.5%	0.03%
Human RNases 2 and 3[9d]	100%	1240%	0%
HSA	100%	2.2%	0.9%

[a] The activity of each protein in the reaction of poly(U) and poly(A) hydrolysis was calculated taken the activity for poly(C) hydrolysis as 100%.

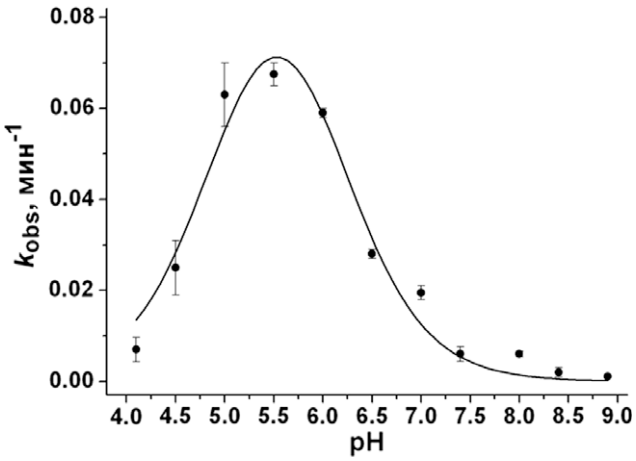

Figure 4. Plots of k_{obs} against pH for the hydrolytic reaction of ON16 with HSA. The data are average values of two independent experiments. Error bars are given in the form of standard deviation. The solid curve is the best fit to the equation $k_{obs} = k/(1+[H^+]/K_1+K_2/[H^+])$, where k is the pH-independent apparent rate constant, K_1 and K_2 are molecular ionization constants for two amino acids side chains of the protein. Reaction conditions: [5′-^{32}P]-ON16 (1×10^{-6} M) was incubated in the presence of albumin (1×10^{-5} M) in a 50 mM buffer, containing 0.2 M KCl, 5 mM EDTA, at 37 °C.

to the substitution of natural HSA by rHSA in critical healthcare situations.

Although albumin's activity in RNA hydrolysis is low in comparison with ribonucleases, the possibility that HSA can participate in extracellular RNAs degradation cannot be excluded, since the concentration of albumin in human blood is rather high (about 0.6 mM).[1a] The discovered activity of HSA may have an important biological role. For instance, RNA-cleavage activity of albumin may act as a defense mechanism against circulating nucleic acid from other (i.e., pathogenic) organisms. Another hypothesis is that albumin may participate in metabolism of endogenous extracellular RNA. From a pharmaceutical point of view, the RNA cleavage activity of HSA should be taken into account when designing therapeutic siRNA. Albumin, along with other blood RNases, can cleave siRNAs, thus reducing their therapeutic effect.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.12.095.

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