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Precipitation of human serum albumin from yeast culture liquid at pH values below 5

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ABSTRACT

In vivo and *in vitro* experiments showed that human serum albumin (HSA) co-precipitated with components of the commonly used yeast peptone dextrose (YPD) growth medium in aqueous solutions at pH <5. Yeast extract was found to be the primary component of YPD responsible for HSA precipitation. Among yeast extract constituents, RNAs are likely to be most important for HSA precipitation. HSA precipitation at pH <5 was reversible, so that HSA was easily re-solubilized by increasing pH above 6 with completely retained immunoreactivity. The co-precipitation and re-solubilization of HSA were solely pH-dependent and occurred almost instantly at room temperature. Practical aspects of the observed HSA co-precipitation are discussed.

Introduction

Human serum albumin (HSA)[1] is a pharmaceutical product with growing demand [1]. HSA can be obtained, in particular, using heterologous expression in different yeast species [2]. HSA expression in yeast is complicated by its partial degradation by yeast proteinases [3–5].

In this paper, we studied HSA stability in culture liquid during yeast growth under conditions favorable for proteolytic degradation. For this purpose, HSA was added to growth medium of a non-HSA-producing yeast strain. Using this system together with a collection of proteinase-deficient mutants, we aimed on identification of additional genes encoding proteinases responsible for HSA degradation.

Unexpectedly, it was found that, under experimental conditions used, exogenous HSA almost completely disappeared from culture liquid and then re-appeared in the initial amounts depending on pH alterations of the growth medium. The observed HSA behavior could be explained by reversible interaction of HSA with some components of the growth medium.

Materials and methods

Strain, media, culture conditions, reagents

A strain *Saccharomyces cerevisiae* B0 (MAT*a* *his3-Δ1*, *his4-519*, *leu2-3,112*, *trp1-289*, *ura3-52*; this laboratory) does not secrete HSA, but can be used as a host for vectors expressing HSA and other recombinant proteins. To grow the B0 strain in the presence of exogenously added HSA, the YpD medium (1% yeast extract, 0.2% peptone, 2% glucose) was used, which differed from the conventional yeast growth medium, YPD, by a 10-fold reduction in peptone content. Preliminary experiments revealed that the YpD medium had a smaller buffer capacity and could be easily acidified to pH values shown to be favorable for proteinase activity [4,5]. Cells were cultured under continuous shaking (250 rpm) at 30 °C.

Yeast extract and peptone used for growth media preparation as well as for *in vitro* experiments were purchased from Serva Electrophoresis GmbH and BD-Difco, respectively. HSA (Sigma–Aldrich) was used as a filter-sterilized aqueous stock solution (25 mg/ml). All experiments with HSA, except cell culturing, were carried out at room temperature. Yeast tRNA (Sigma–Aldrich) and ATP (Serva Electrophoresis GmbH) were used as aqueous stock solutions at 10 mg/ml and 100 mM, respectively.

Analytical methods

HSA concentrations were determined in cell-free samples. HSA assay was carried out either according to Bradford [6] using bovine serum albumin (Sigma–Aldrich) as a standard or with ELISA using A1274-85 kit (US Biological) and a protocol recommended by the manufacturer.

For protein separation, SDS–PAGE in 10% gels and Coomassie Brilliant Blue staining were used. Nucleic acids were separated using electrophoresis in 1% agarose gel in TAE buffer followed by ethidium bromide staining.

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[1] *Abbreviations used:* HSA, human serum albumin; buffer, 20 mM citrate-phosphate buffer pH 7.

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Results

Evidence for HSA precipitation

When the B0 strain was grown in YpD in the presence of exogenous HSA, detectable HSA concentration in cell-free culture liquid varied depending on sampling time and pH values of the growth medium (Fig. 1). After almost complete disappearance by 16 h of cultivation, HSA could be recovered at 24 h in the initial amounts, as revealed by both ELISA and SDS–PAGE. This HSA recovery ruled out the possibilities that HSA disappearance from the culture liquid was caused by either proteolytic degradation or irreversible protein denaturation. Most likely, HSA was eliminated from the growth medium during cell pelleting, a step of cell-free sample preparation for the HSA assay. And indeed, HSA was detected in 20 mM citrate–phosphate buffer pH 7 (buffer) used to dissolve pellets in samples, which were withdrawn after cultivation for 16 and 40 h (data not shown). Apparently, this HSA removal was either growth phase-dependent or pH-dependent. When buffered YpD medium was used with a fixed pH value, no changes in HSA concentration in culture liquid were observed throughout cultivation period up to 40 h. The data suggest that it was medium acidification, rather then culture age, which caused HSA elimination.

Therefore, the term "HSA precipitation" used below means transition of soluble HSA to the insoluble form, which is eliminated from growth medium during sample centrifugation at 14,000*g* for 1 min performed to spin down cells.

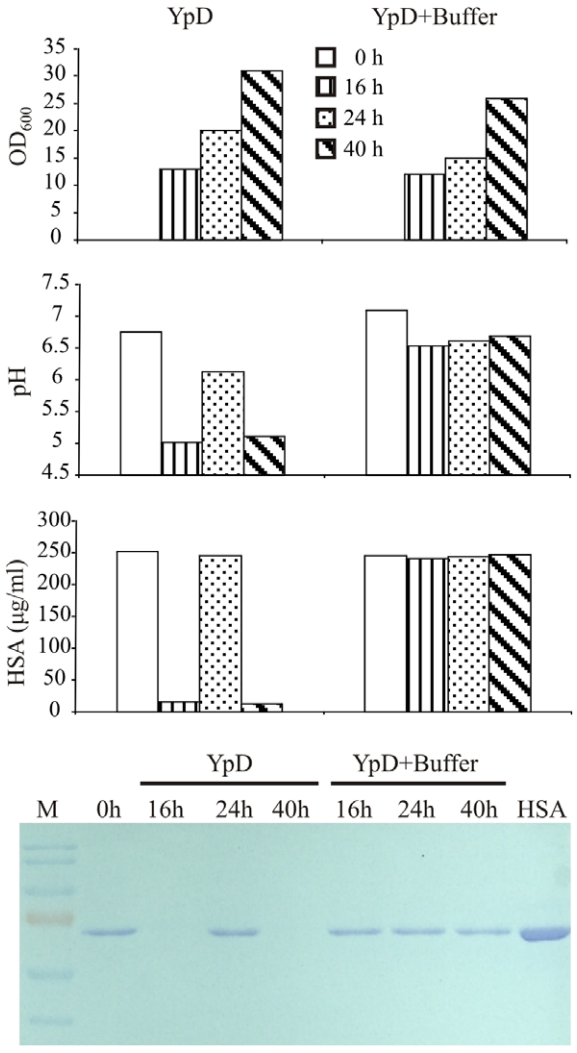

Fig. 1. Kinetics of cell growth (OD_{600}), pH of growth medium, and HSA concentration during cultivation of the B0 strain in YpD (YpD) or YpD prepared in buffer (YpD + buffer). HSA was added at the zero time point to a final concentration of ~250 μg/ml. HSA in the culture liquid was either determined quantitatively using ELISA or revealed qualitatively using SDS–PAGE (see electrophoregram in the lower part of Fig. 1: M, molecular mass markers; HSA, HSA marker; equal volumes of samples were loaded for each lane).

HSA precipitates with components of growth medium but not with cells

The simplest explanation of the observed HSA disappearance from culture liquid could be HSA adsorption on cell surface followed by cell pelleting.

The results of *in vitro* experiments (Fig. 2) showed that HSA concentrations in the presence of washed B0 cells did not change substantially within pH range from 3 to 7. At the same time, in the unseeded YPD medium and in cell-free or cell-containing culture liquid of the B0 strain, concentration of water-soluble HSA changed in a pH-dependent manner. Similar results were obtained when acetic or hydrochloric acids were used in place of citric acid for acidification (data not shown).

The results presented in Fig. 2 clearly indicate that HSA alone (i.e. in the absence of growth medium components) did not precipitate at pH values below 5 (see curve 2 in Fig. 2). On the other hand, HSA precipitation at low pH was not caused by HSA interaction with yeast cells (see curves 3–5 in Fig. 2). Thus, the most plausible explanation of the above results is that HSA precipitation at pH <5 was due to HSA interaction with certain component(s) of the YPD medium.

The similarity of curves 1, 3, and 4 in Fig. 2 suggests that YPD components interacting with HSA and existing both in fresh and conditioned media were expended quite little for cell growth and remained in excess relative to added HSA amounts (i.e. were present in concentrations, not limiting their co-precipitation with HSA) even at the stationary phase of cell growth. Since glucose is known to be consumed rapidly as cells are grown, yeast extract and/or peptone could be the only potential sources of YPD constituents interacting with HSA.

Analysis of the YPD components

The assumption that yeast extract and peptone could be involved in HSA precipitation was tested directly. The results pointed

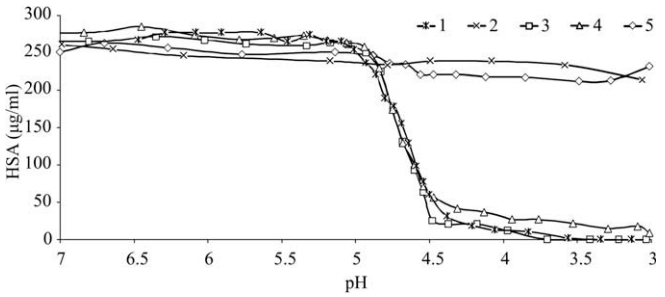

Fig. 2. HSA concentrations in different media during progressive acidification. The following media were tested for HSA content: YPD (curve 1: ж); buffer (curve 2; ×); cell-free culture liquid of the B0 strain grown in YPD until the stationary phase (curve 3; □); cell-containing culture liquid of the B0 strain grown in YPD until the stationary phase (curve 4; △); in-buffer suspension of washed B0 cells (10^8 cells/ml) grown in YPD until the stationary phase (curve 5; ◇). HSA was added to a final concentration of ~250 μg/ml with pH = 7. Acidification of the tested medium was done by adding 10–100 μl portions of 0.2 M citric acid to the continuously stirred medium, the steady-state pH value was measured, and an aliquot of the sample was taken for analysis. Then acidification was continued. The samples were centrifuged for 1 min at 14,000*g*, and HSA concentration was determined in the supernatants by Bradford method. The same procedure was used in other experiments shown in Figs. 3, 4 and 6.