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PURIFICATION OF PIG AND RAT LIVER SQUALENE EPOXIDASE BY AFFINITY CHROMATOGRAPHY

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Abstract. Three novel affinity columns were evaluated for purification of rat and pig squalene epoxidase (SE). Pig SE was adsorbed to a resin bearing an *N*-cyclopropyl-*N*-trisnorsqualenamine (TNS-CPA) type functionality, and eluted with 25 mM KCl to give a 55-56 kDa doublet, whereas rat SE required the use of *N*-methyl-*N*-trisnorsqualeneamine (TNS-MA) affinity resin. Rat SE could be eluted with 0.5M KCl, to give a single band at 52 kDa.

Squalene epoxidase (SE) (EC 1.14.99.7) catalyzes the conversion of squalene to (3*S*)-2,3-oxidosqualene.[1] This reaction and the subsequent cyclization of (3*S*)-2,3-oxidosqualene by oxidosqualene cyclase (OSC) to lanosterol are the key steps in cholesterol biosynthesis in vertebrates.[2,3] SE has been the focus of efforts to develop hypocholesterolemic, herbicidal and antifungal agents.[4,5] To this end, we have developed a method of purifying vertebrate SE by affinity chromatography, which makes use of the very strict substrate requirements for SE to achieve bioselective adsorption. Potent slow, tight-binding inhibitors are used to achieve bioselective elution.

MATERIALS AND METHODS

Enzyme Preparations. Rat liver SE was partially-purified by the protocol developed by Ono.[6,7] Pig liver microsomal SE was partially purified by modification of the tandem DEAE/Blue Sepharose procedure.[8] Solubilized microsomes were first applied to a Sephadex G-25 column (4.8 cm x 28 cm) and the flow-through was then applied to the DEAE-Sephacel column as previously described. Protein concentrations were measured for all steps by the modified Lowry method. The partially-purified SE activity was assayed by radio-TLC using [^{14}C]squalene as substrate[8], except that the assay volume was 200 µl and the substrate was dissolved in 2 µl of 0.5% Tween-80 in ethanol; the assay time was 50 min, but results are reported for conversions in nmol/h.

Preparation of 26-Aminosqualene Affinity Resin. 26-Aminosqualene (26-AS) was prepared by X.-y. Xiao as previously described.[9] Affi-Gel 10 (2.78 g, Pierce Chemical Co.) was suspended in 5 ml of 0.1 $NaHCO_3$, pH 8.4. Next, 26-AS (5.2 mg) dissolved in 16 ml isopropanol was added, and the resulting mixture was diluted with 10 ml of H_2O. The slurry was mixed for 18 h at 4 °C. Then, three drops of 2-(aminoethanol) in 10 ml of 0.1 M $NaHCO_3$, pH 8.4, was added and mixing was continued for an additional 20 h. The 26-AS affinity resin was then washed (2 x 10 ml THF, 2 x 10 ml H_2O) and stored at 4 °C. Using this method, coupling of the ligand to the resin was quantitative.

Preparation of TNS Affinity Resins. Hexanorsqualene bisaldehyde was prepared as previously described.[10,11] ω-Aminohexyl agarose (3 ml in 0.5 M NaCl, Sigma Chemical Co.) was suspended in 20 ml of THF. Bisaldehyde (50 mg), sodium cyanoborohydride (9 mg), and acetic acid (8 mg) were added to the slurry, and the pH was adjusted to 6.0 with 5 M NaOH. The slurry was mixed for 24 h at 4 °C; then, excess cyclopropylamine or methylamine-HCl (10 eq.) was added, and the pH of the slurry was adjusted to 6.0 with 10% acetic acid. The slurry was mixed for 24 h at 4 °C, and washed with ethanol/H_2O. The coupling efficiency of bisaldehyde to the solid support was assessed by analyzing the ethanol/H_2O wash by TLC in 20% ethyl acetate/hexane. Using this method, we found that 100% of the starting material was bound to the solid support, and therefore, was converted to the corresponding bound inhibitor form. Unused resin was stored at -20 °C, in 5 mM potassium phosphate buffer, pH 8.8.

Affinity Purifications. For the 26-AS resin, the Blue Sepharose fraction with SE activity (3 ml) was incubated with 5.6 ml of 26-AS resin for 14 h at 4 °C. The resin was then placed in an Econoflow column and was washed with 20 mM Tris-HCl, pH 7.4 (buffer A) for 10 min (9 ml/h). SE activity was recovered by elution with 1.0 M KCl in buffer A. Subsequently, SE activity was eluted with TNS-CPA, a potent slow, tight-binding inhibitor of SE.[12] The eluate was adsorbed to a DEAE column pre-equilibrated with buffer A, and the TNS-CPA was eluted with the flow-through. SE activity was then eluted with 200 mM KCl.

The TNS-CPA resin (0.5 ml) was diluted with ten volumes of Sepharose CL-6B. This was pre-equilibrated with SE (1 ml, 2 mg protein) for up to 18 h at 4 °C with stirring. The flow-through (3-5 ml/h) was re-applied five times. Purified SE was selectively eluted with 25 mM KCl or 725 μM TNS-CPA in 5 mM KPB, pH 8.8, containing 1 mM EDTA, 1 mM DTT, 10% glycerol and 0.1% Triton X-100. Purified pig SE could be eluted from the resin using 20 mM Tris-HCl, pH 8.8 as the buffer using Triton X-100 concentrations from 0.05% to 1%. Elution was relatively insensitive to the quantity of Sepharose CL-6B that was used for dilution of the resin. For rat SE, the TNS-MA resin was diluted with an equal volume of Sepharose CL-6B, and was used as described for TNS-CPA resin. Purified rat SE was selectively eluted with 0.5 M KCl in 5 mM KPB, pH 8.8, containing 1 mM EDTA, 1 mM DTT, 10% glycerol and 0.1% Triton X-100. Selective elution of rat SE was only obtained at this detergent concentration and this ratio of Sepharose CL-6B to affinity resin. We found no appreciable difference in the SE yields for elution with TNS-CPA versus a salt gradient.

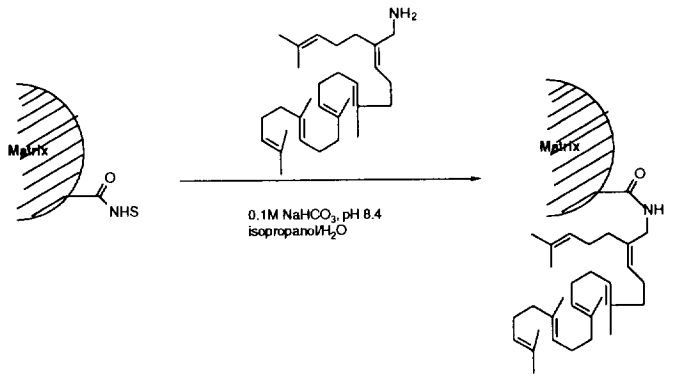

Figure 1. Synthesis of a 26-AS affinity resin for SE

RESULTS AND DISCUSSION

The prototype affinity resin for SE purification was based on the observation that selected derivatives of 26-hydroxysqualene (26-HS) were both inhibitors of, and substrates for, partially-purified liver SE.[9,13] We reasoned that these substrate analogs would be bound at the active site while still providing a position for attachment to a linker chain appended to a solid support. The allylic amine 26-AS (IC_{50} values for pig SE = 32 μM at pH 8.8, 109 μM at pH 7.4) was readily coupled to an NHS-activated ester resin, Affi-Gel 10 (Figure 1).

TNS-CPA was known to be a slow, tight-binding inhibitor of pig SE with a $K_i = 2.4$ μM at pH 7.4.[12] To obtain a TNS aldehyde-like species immobilized on a solid support, we used the reductive coupling of a bisaldehyde to an aminoalkyl-activated resin. We reasoned that once the precursor was attached to the resin via the first aldehyde, if the probability of crosslinking could be kept low, then the second reductive amination with cyclopropylamine would generate the desired TNS-CPA resin. This strategy proved highly successful (Figure 2). Similarly, a mimic of *N*-methyl-*N*-trisnorsqualenamine (TNS-MA), an inhibitor of rat SE with an IC_{50} of 1.0 μM (B.A. Madden and P. Denner-Ancona, unpublished results), could be attached to a solid support via the bisaldehyde using reductive amination with methylamine.

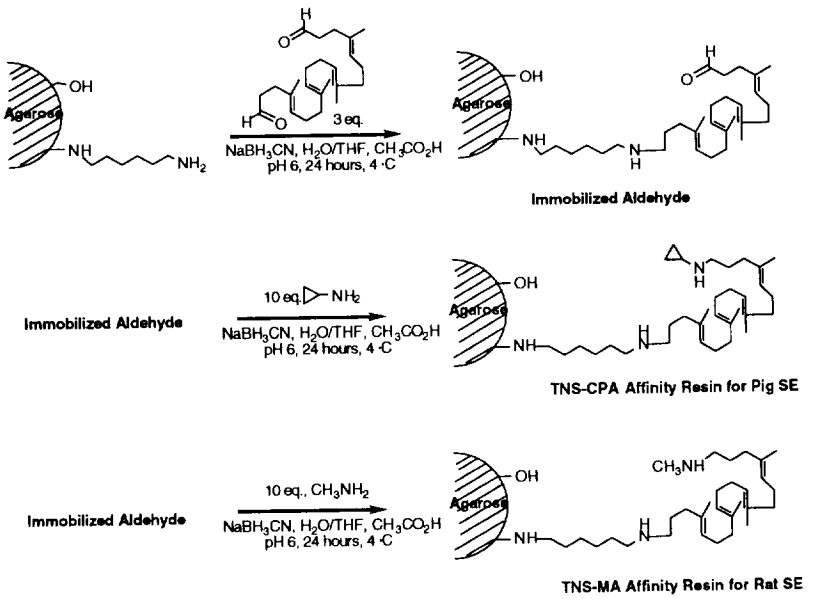

Figure 2. Synthesis of amine-terminated affinity resins for purification of pig SE and rat SE.

Simple hydrophobic resins such as Phenyl Sepharose or custom-made dodecylthiol or benzylthiol coupled to epoxy-activated Sepharose CL-6B failed to retain SE activity (M. Bai, unpublished results). However, the 26-AS resin retained pig SE activity effectively, and SE could be eluted with 1 M KCl. The TNS-CPA resin also retained pig SE activity effectively, and the activity could be eluted at 25 mM KCl, a lower salt concentration than

that required for elution from the 26-AS resin. A net 116-fold purification was achieved in this very rapid sequence. Importantly, bioselective elution of highly-purified, active SE was achieved when high salt or TNS-CPA was used as the eluent with the 26-AS and TNS-CPA resins. This inhibitor could be removed from the active SE fractions and SE activity could be regenerated using a DEAE column. We found that there was no advantage to using TNS-CPA as the eluent versus a salt elution, as there was no marked increase in the yield or reproducibility. Note that the SE activity was higher in purified SE, since traces of an endogenous inhibitor present in microsomes was removed during purification. Denaturing polyacrylamide electrophoretic gels illustrating the protein homogeneity at each stage are shown in Figure 3.

When rat SE was used with the TNS-CPA resin, selective adsorption to the resin was not observed. For this reason, the TNS-MA resin was prepared. The TNS-MA resin retained the rat SE activity effectively, and the enzyme could be eluted with 0.5 M KCl. The resulting fractions collected from this protocol were homogeneous, as they contained a single band at 52 kDa. Figure 3 shows the relative level of purity of the rat liver SE after affinity chromatography. It is interesting to note that the pig SE appears as a doublet, suggesting that isoforms of the enzyme may exist. However, in the preparation of the rat enzyme, this doublet, which is evident at the penultimate stage of purification, can be separated by careful manipulation of the separation conditions. We have not yet determined whether both protein bands of the doublet possess SE activity.

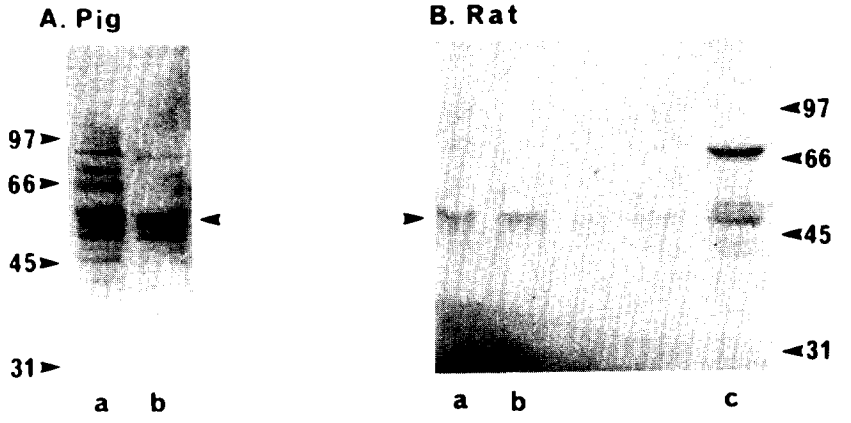

Figure 3. Relative purification of rat and pig squalene epoxidase.
Panel A shows the pig SE: **lane a,** DEAE/Blue-Sepharose fraction; **lane b**, affinity-purified SE.
Panel B shows the rat SE: **lane a,** affinity-purified SE; **lane b,** DEAE/Blue-Sepharose fraction.

Table 1. Purification Table for Pig Liver Squalene Epoxidase

	mg protein	*nmol/h*	*nmol/h/mg*	*% yield*	*Fold*
Solubilized microsomes	401	85.4	0.213	100	1
DEAE/Blue	25	115.9	4.64	135	21.7
TNS-CPA	5	123.6	24.7	144	116

Table 2. Purification Table for Rat Liver Squalene Epoxidase

	mg protein	*nmol/h*	*nmol/h/mg*	*% yield*	*Fold*
DEAE/Blue	8.1	133.3	16.5	100	1
TNS-MA	1.6	96.6	60.4	72	3.64

Squalene epoxidase requires squalene as the substrate and oxygen, flavin adenine dinucleotide (FAD), cytochrome P-450 reductase, NADPH, and a soluble protein factor (SPF) for activity.[6,14,15] SPF, a 47-kDa protein, has been purified and has been shown to be involved in intermembrane squalene transport.[14,16,17] In enzyme assays, SPF may be replaced by 0.1% Triton X-100. SE is the only non-cytochrome P-450 enzyme of note that epoxidizes an alkene; moreover, it selects only a single enantiotopic face of one of six trisubstituted olefins. Rat liver SE has been purified from Triton X-100-solubilized microsomal proteins using a six-step procedure and showed a subunit size of 52 kDa.[6,18] The rat enzyme showed no distinct absorptions in the visible region and no CO-induced effects that would be diagnostic for P-450 isozymes. It was insensitive to SKF 525A, metyrapone, or other standard cytochrome P-450 inhibitors; cyanide, CO, azide, catalase, superoxide dimutase, and other modifiers of oxygen reduction products also failed to alter SE activity.[6]

Pig liver SE was first purified by a tandem DEAE/Blue Sepharose protocol and employed for extensive studies on substrate, activator, and inhibitor kinetics.[8] SE has extremely strict steric and electronic requirements for epoxidation. The intact polyolefinic squalene chain appears necessary for epoxidation to occur, and (among the squalenoid inhibitors) truncation or elongation of the trisnorsqualene chain generally diminishes inhibitory potency.[10,19] Many unsolved mechanistic questions have been left unanswered by a wealth of confusing data concerning SE. Such results include those for the pH dependency of pig SE, the activation of pig SE by farnesoyl derivatives[8], the requirement of pig SE for the FAD cofactor, and the differential sensitivity to TNS and allylamine inhibitors for conversion to the oxidosqualene from the bisepoxide by pig and rat SE.[13] Access to highly-purified SE has limited progress in answering these questions.

In this paper, we have demonstrated the inhibitor-based affinity purification of two vertebrate SEs having quite different properties. Rapid and species-selective purifications of this enzyme have been elusive for over two decades, and this methodology provides a route to accelerate progress on SEs from a variety of species. We have also shown that both the rat and pig SE appear as doublets, suggesting that isozymes of SE may be present in both species. Characterization of the separate proteins present in affinity-purified rat and pig SEs are in progress.

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