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Subunits of neurosteroid sulfatase from bovine brain

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Abstract

We have purified the neurosteroid sulfatase (NSS) from Triton X-100 solubilized microsomes of bovine brain about 100-fold. The purified enzyme is composed of two catalytic units (MW: 57 kDa) and two regulatory units (MW: 38 kDa), making it an $\alpha_2\beta_2$ heterotetramer, whose apparent molecular weight was 180 kDa by gel filtration in the presence of Triton X-100. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

Neurosteroids are known to exert their regulatory effects on the central nervous system through $GABA_A$ receptor in a bimodal fashion. 5α-Pregnan-3α-ol-20-one (tetrahydroprogesterone; THP), 21-hydroxy-THP and androsterone are $GABA_A$-agonist [1–5], whereas 3β-hydroxy-5-pregnen-20-one sulfate (pregnenolone sulfate; Preg-S) and dehydroepiandrosterone sulfate (DHEA-S) are antagonists by non-competitive binding to the $GABA_A$receptor [6,7].

Steroid sulfatase in the brain converts Preg-S and DHEA-S into Preg and DHEA, respectively. Thus, it is highly likely that neurosteroid sulfatase plays a key role in the regulation of $GABA_A$ receptor [8,9]. Although steroid sulfatases from various species and tissues have been characterized [10–14], neurosteroid sulfatases (NSS) in the brain have been little studied. We recently reported some enzymatic characteristics of NSS in microsomes of bovine midbrain [15] and in Triton X detergent solubilized state [16]. In this paper we report the purification of NSS from bovine brain to apparent homogeneity and some characteristics of this enzyme.

2. Materials and methods

2.1. Materials

Bovine brain of 2- to 2.5-years-old females was obtained within 2 h of death and transported on ice. Dehydroepiandrosterone sulfate (DHEA-S; 1 mCi/mg) was obtained from New England Nuclear Corp. (USA), and the Triton X-100 was from Sigma (USA). All other chemicals were of analytical grade.

2.2. Solubilization of microsomal proteins

The procedure for preparing a microsomal fraction from bovine midbrain was previously described [15]. To solubilize the microsomal proteins prepared by the method previously described [16], 6 mg of microsomal proteins suspended in 300 μl 10 mM Tris–HCl buffer, pH 7.4, containing 0.32 M sucrose was mixed with 200 μl of a 1.5% solution of Triton X-100. The final volume was adjusted to 1 ml with Tris–HCl buffer. The mixture was gently agitated at 4°C for 60 min and centrifuged at 105,000 × *g* for 60 min. The supernatant

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Table 1
Purification of neurosteroid sulfatase from bovine midbrain microsomes

Treatment	Total protein (mg)	Total activity (pmole/h)	Specific activity (pmole/mg/h)	Yield (%)	Purification (fold)
Microsomes	2000	3580	1.79	100	1.0
Triton extract	846	5254	6.21	147	3.5
DEAE-cellulose	89	3088	34.70	86.3	19.4
Gel filtration	13	1799	138.40	50.3	77.3
SDS-PAGE[a]	3	533	177.70	14.9	99.3

[a] Reconstituted enzyme.

was recovered for purification and enzyme assay. The protein concentration was determined by the method of Lowry et al. [17]. Since Triton X-100 interferes with protein determination by forming precipitates, the same amount of the detergent was systematically added to the standard solutions. The solutions were centrifuged at 3000 × *g* for 10 min before measuring their absorption.

2.3. Chromatographic procedures

The extract was concentrated by ultrafiltration using a Centricon membrane filter with a molecular weight cut-off of 30 kDa (Amicon, USA). Two hundred milligrams of protein was applied to a DEAE-cellulose column (2.6 cm × 40 cm) already equilibrated with Tris–HCl (10 mM, pH 7.4) containing 1 mM EDTA, 0.3% Triton X-100 (TET buffer). The column was washed with 150 ml of TET buffer, and the NSS was eluted with a linear gradient of KCl from 0.0 to 0.3 M in TET buffer. Fractions of 5 ml were collected. The fractions showing NSS activity were pooled and dialyzed overnight against TET buffer. After concentration with ultrafiltration, 5 mg of protein was applied to a Sephadex G-150 column (1.0 cm × 150.0 cm) previously swollen in the same buffer. Fractions of 4 ml were collected at a rate of 0.5 ml/min with the same buffer. The fractions corresponding to NSS were pooled and concentrated with ultrafiltration.

2.4. Preparative gel electrophoresis

Preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [18]. The concentrated NSS fraction was separated in 15% SDS-PAGE. Electrophoresis was done at 4°C for 6 h at constant voltage of 200 V. The bands corresponding to the subunits were cut out, homogenized, and centrifuged at 20,000 × *g* for 30 min. The supernatant thus obtained was dialyzed for 48 h against TET buffer. Continuous-elution electrophoresis (Mini-Prep Cell, Bio-Rad, USA) was carried out at 200 V with 5–10% Laemmli gels. The gel was electrophoresed at 4°C and the elution buffer (25 mM Tris–192 mM glycine, 0.1% SDS) was collected at a rate of 1.5 ml/20 min.

Native gel electrophoresis was done according to the method described by Davis [19].

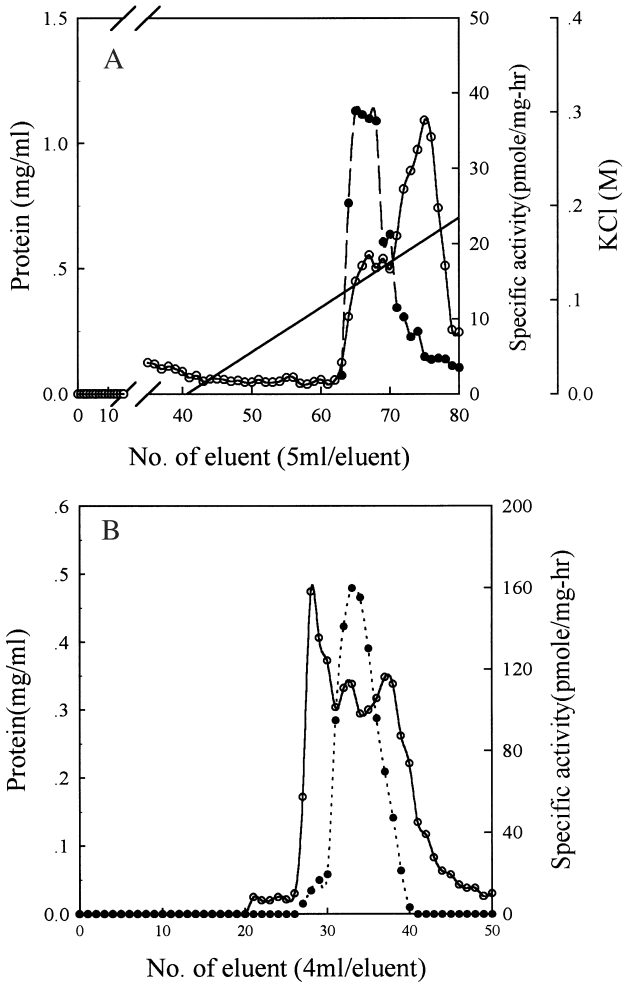


Fig. 1. Liquid chromatography of solubilized NSS. (A) DEAE-cellulose column chromatography. Solubilized microsome was applied to a DEAE-cellulose column and eluted with a linear gradient of KCl. -○-, Protein; -●-, neurosteroid sulfatase activity; - -, KCl gradient. (B) Sephadex G-150 gel filtration. The pooled NSS fraction from the DEAE-cellulose column was applied to column of Sephadex G150 and eluted with TET buffer. -○-, Protein; -●-, neurosteroid sulfatase activity.

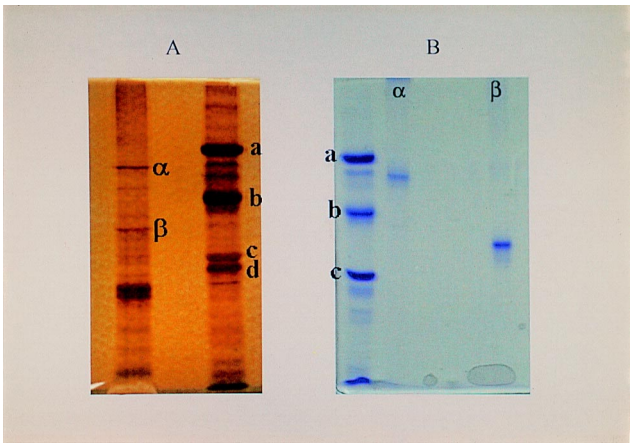


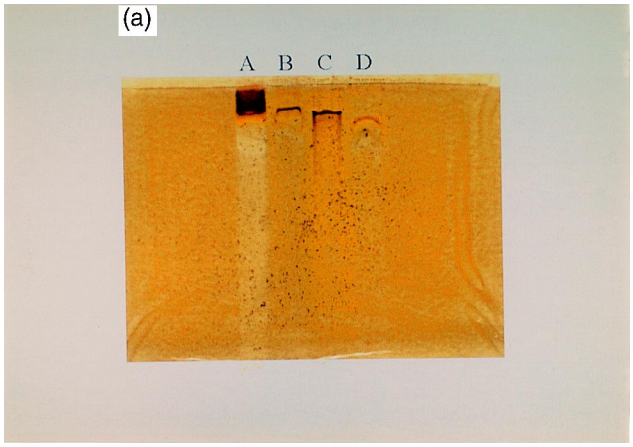
Fig. 2. SDS-PAGE of partially purified NSS (α and β subunits) and purified α and β subunits. (A) SDS-PAGE of fraction 33 of gel filtration chromatography. Fraction 33 has the highest activity of NSS. (B) SDS-PAGE of NSS subunits purified by preparative SDS-PAGE. α: α subunit; β: β subunit. Each enzyme preparation was electrophoresed in 10.0% polyacrylamide gel in the presence of 0.1% SDS. Molecular markers; a: Albumin, bovine (66 kDa). b: Albumin, egg (45 kDa). c: Carbonic anhydrase(29 kDa) d: Tryspinogen (24 kDa).

2.5. Enzyme assay

The method for determining NSS enzyme activity was described previously [15]. Briefly, the reaction mixture contained 46 nM [7−^{3}H]-DHEA-S (23 pmole) and appropriate amount of the enzyme in a total volume of 0.5 ml 10 mM Tris–HC1 buffer (pH 7.4). After incubation at 37°C for 1 h, freezing at −70°C stopped the reaction. Two milliliters of ethyl acetate-isooctane mixture (1:1, v/v) was added to the incubation mixture, and the freed steroid was extracted into the upper phase. After three extractions with the organic solvent, the radioactivity of free [7−^{3}H]-DHEA was measured with a liquid scintillation counter (1600TR liquid-scintillation counter, Packard, USA).

3. Results and discussion

The use of Triton X-100 not only proved to be highly efficient in releasing NSS, but also caused apparent activation (Table 1.). Although this activation is partly due to the elimination of potential inhibitors in the microsomes and also due to the proper action of Triton X detergents [16], the precise mechanisms responsible for this apparent activation remained to be elucidated. However, there have been a substantial number of examples that some nonionic and amphoretic detergents have been shown to activate steroid sulfatases [16,20,21]. In DEAE-cellulose chromatography, NSS was eluted at a KCl concentration of 0.13 M with a yield of 86% (Fig. 1A). Gel filtration separated this 0.13 M KCl fraction into three major protein peaks (Fig. 1B). The pooled fractions containing higher NSS activity were further purified with preparative SDS-PAGE (Fig. 2A). The purified subunits



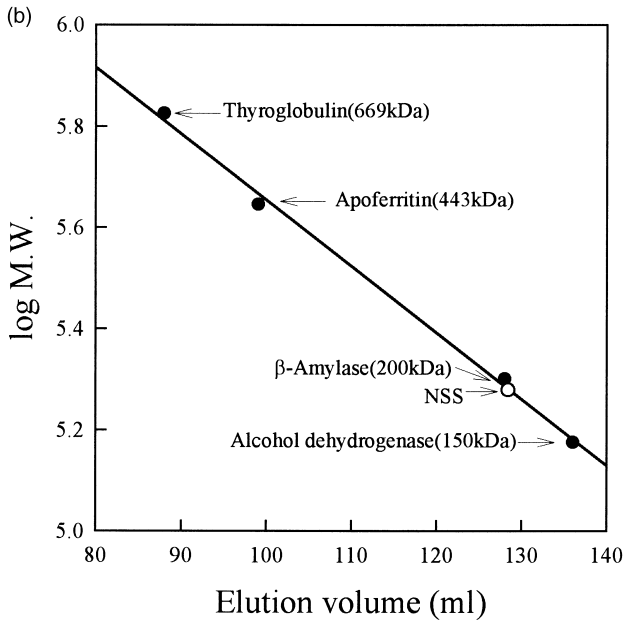


Fig. 3. Native-PAGE and gel filtration of the reconstituted NSS. (a) Native-PAGE of the reconstituted NSS. Each protein was electrophoresed in 7.5% acrylamide gel without SDS. A: F33 of Sephadex G-150. B: Reconstituted NSS . C: α subunit. D: β subunit. (b) Determination of apparent molecular weight of the reconstituted NSS by gel filtration.

Table 2
Enzyme activity of the components of neurosteroid sulfatase

Fraction or protein band	Fraction of gel filtration	α Subunit	β Subunit	α + β Subunit
Relative enzyme activity (%)[a]	100	5	0	130

[a] The specific activity of NSS before SDS-PAGE was taken as 100% and that of each subunit was expressed in relative activity.

(α and β subunits) were apparently homogeneous, as judged by polyacrylamide gel electrophoresis in the presence of SDS (Fig. 2B). Based on the relative mobility of standard proteins, the α and β subunit of NSS observed in denaturing SDS-gels migrate with apparent molecular weights of 57 kDa and 38 kDa, respectively (Fig. 2B). However, the β subunit showed only 5% of the original activity toward the substrate, while the β subunit was inactive (Table 2). In contrast, a full recovery of the NSS activity was obtained upon combining both fractions (Table 2). As seen in Table 1, the reconstituted NSS was purified about 100-fold from microsomes, with an overall yield of 15%. Upon reconstitution this NSS showed a single band in non-denaturing electrophoresis (Fig. 3A). The relative molecular weight of the reconstituted NSS was estimated to be 180 kDa by gel filtration (Fig. 3B). The reconstituted form of NSS is likely to be an α_2 β_2 heterotetramer. NSS is composed of a catalytic unit (α subunit) and a regulatory unit (β subunit).

Detergents can vary the molecular weight of detergent-solubilized proteins. The apparent molecular weight of arylsulfatase C from human cultured fibroblasts was 85 kDa in the presence of Triton X-100 and 335 kDa in the presence of an amphoretic detergent Miranol [20]. Arylsulfatase C from rat liver microsomes is a tetrameric enzyme, with a molecular weight of 280 kDa in the presence of Triton X-100 [22].

A steroid sulfatase from murine liver was reported to be a homodimeric enzyme with different degree of glycosylation on the same molecule [23]. The purified enzyme had an apparent molecular weight of 128 kDa on gel filtration, whereas the enzyme migrated as 60 and 68 kDa on SDS-PAGE.

Recently an estrone sulfatase purified from human placenta microsomes was shown to be composed of catalytic and regulatory units [24]. The activity of the catalytic unit of the estrone sulfatase without the regulatory unit was as high as that of the combined enzyme. Combining the regulatory unit with the catalytic unit modifies the enzyme kinetics without changing V_{max}. In this context, the regulatory unit (β subunit) of NSS seems to be different from its estrone sulfatase homologue, because the NSS showed 5% of its original activity without β subunit. In conclusion, the purified bovine brain NSS is a different type of allosteric steroid sulfatase. Further characterization of its subunits to obtain more detailed information is now under way.

Acknowledgements

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