INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

U·M·I

University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600

Order Number 9119692

Purification and characterization of a neuronal nicotinic acetylcholine receptor from *Drosophila melanogaster*

Wu, Peipei, Ph.D.

City University of New York, 1991

Copyright ©1991 by Wu, Peipei. All rights reserved.

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

NOTE TO USERS

THE ORIGINAL DOCUMENT RECEIVED BY U.M.I. CONTAINED PAGES WITH SLANTED AND POOR PRINT. PAGES WERE FILMED AS RECEIVED.

THIS REPRODUCTION IS THE BEST AVAILABLE COPY.

A

Purification and Characterization of A Neuronal Nicotinic Acetylcholine Receptor from Drosophila melanogaster

by

Peipei Wu

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1991

Peipei Wu

All Rights Reserved

1991

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

1/18/91
date

Thomas Schmidt-Glenewinkel
Chairman of Examining Committee

1/29/91
date

Executive Officer

Thomas Schmidt-Glenewinkel
Dr. Thomas Schmidt-Glenewinkel, Hunter

Dr. Joseph Krakow, Hunter

Dr. Robert Dottin, Hunter

Sally Hoskins, CCNY
Dr. Sally Hoskins, City

L. Pick
Dr. Leslie Pick, Mt. Sinai

Supervisory Committee

The City University of New York

Abstract

Purification and Characterization of A Neuronal Nicotinic Acetylcholine Receptor from Drosophila melanogaster

by

Peipei Wu

Adviser: Professor Thomas Schmidt-Glenewinkel

The central nervous system of Drosophila contains an α-Bungarotoxin binding activity with the properties expected of a nicotinic acetylcholine receptor. The receptor was purified 5800-fold by affinity chromatography. The receptor has a $S_{20,w}$ of approximately 9.5 and a Stoke's radius of 7.4 nm. The frictional coefficient was calculated to be 1.7, indicating a highly asymmetrical protein structure. From sedimentation analysis in H_2O and D_2O, a molecular weight of 270,000 dalton was determined. SDS polyacrylamide gel electrophoresis followed by silver staining revealed two subunits with apparent molecular weights of 44,000 and 57,000 daltons. Polypeptides of the same size were identified by photoaffinity labeling of the crude membrane fraction in situ and of the purified receptor protein. The individual polypeptides were blotted onto a nitrocellulose membrane, digested with trypsin, separated by microbore reverse phase HPLC and subjected to internal amino acid sequence analysis. Screening of a cDNA library from Drosophila with oligonucleotide probes derived from the amino acid sequence allowed identification of several cDNA clones.

Acknowledgements

I wish to acknowledge those people who have made this thesis and my graduate training possible. My greatest thank goes to my mentor Dr. Thomas Schmidt-Glenewinkel for sharing with me his dedication to science , for his patience in my scientific training, for forbidding me to speak Chinese in the lab and for his care for a student from a different cultural background. Without his effect, this achievement would have been clearly impossible.

I wish to give my sincere thanks to Steve Bobin for all the help; to my lab colleagues Xiaoping Mai, Xiaoying Zhang, Lee-Chuan Yang, Jun Wu, Harvey Meones and Clara Paulhiac for their contribution in raising flies, for shared works and companionship, especially to Xiaoying Zhang and Xiaoping Mai who were tragically killed in a car accident.

I wish to thank sincerely the members of my thesis advisory committee Drs. Joseph S. Krakow, Robert Dottin, Leslie Pick and Sally Hoskins; the professors and friends at Hunter College for all the help I received from them during the years of my training.

I wish to thank sincerely my parents in China for their love and self-sacrifice to send their daughter far away with nothing left but missing me and the hope that I shall succeed.

I must again thank Dr. Thomas Schmidt-Glenewinkel to complete this acknowledgement. I will remember forever what he told me one day. "You don't know how difficult it was for me to keep you on track!"

TABLE OF CONTENTS PAGE

PAGE

PAGE

PAGE

List of Figures

PAGE

List of Tables PAGE

Abbreviations

ACh	acetylcholine
AChR	acetylcholine receptor
nAChR	nicotinic acetylcholine receptor
α-Bgt	α-Bungarotoxin
α-Cbt	α-Cobratoxin
GABA	γ amino butyric acid
mw	molecular weight
CNS	central nervous system
mAb	monoclonal antibody
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
PIM	protease inhibitors mixture
PVP	polyvinyl pyrrolidone
IAA	iodoacetamide
PMSF	phenylmethylsulfonyl fluoride
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.
BSA	bovine serum albumin
TFA	trifluroacetic acid
PTH	phenyl thiohydantoin

INTRODUCTION

Acetylcholine (ACh) is a major excitatory neurotransmitter at many synapses in both vertebrates and invertebrates. At cholinergic synapses, ACh released by presynaptic cell elicits a postsynaptic response by binding to receptors on the postsynaptic cell. Two different types of receptors respond to this neurotransmitter and result in different responses. The muscarinic acetylcholine receptor, which can be selectively stimulated by muscarine, a drug obtained from the fungus Amanita muscaria, is coupled to G proteins and functions via a second messenger system. The nicotinic acetylcholine receptor, which can be selectively agonized by nicotine, an alkaloid from Nicotiana tobacum, functions through forming an integral ion channel .

In vertebrates, nicotinic acetylcholine receptors (nAChR, defined as an ACh gated ion channel) exist both at the neuromuscular junction and in the peripheral and central nervous system. Pharmacological and physiological experiments have clearly shown that the functional characteristics of neuronal nAChRs are substantially different from those of muscle receptors (Conti-Troconi, 1985; Colquhoun et al., 1987). While the nAChR at the neuromuscular junctions is one of the best characterized neurotransmitter receptors (even though, many questions remain unanswered, for reviews, see Maelicke, 1984; Changeux et al., 1987; Claudio, 1989), the studies on nAChR in neuronal systems are much less advanced (Clementi, 1986).

This can be attributed to several reasons: lack of a convenient system as muscle for electrophysiological experiments; lack of a nervous system that provides as much nAChR as electric organs do for biochemical studies, and lack of suitable molecular probes. The neuromuscular nAChRs are readily blocked by very low concentrations of α-Bungarotoxin (α-Bgt), a basic snake venom toxin protein from Bungarus multicinctus. Thus, α-Bgt is a powerful tool for the purification and characterization of muscle type nAChRs However neuronal nAChRs show a different pharmacology from nAChRs at neuromuscular junction. When α-Bgt was used as a probe for neuronal nAChRs, a very complicated picture emerged. It was found that neuronal nAChRs could be pharmacologically divided into several subtypes: antagonized by α-Bgt, binding of α-Bgt without apparent biological effect, and α-Bgt binding sites which may not represent functional nAChR but have some other unknown functions (for review,see Clark, 1987).

In insects, acetylcholine appears to be one of the predominant neurotransmitters in the CNS (Sattelle, 1980; Restifo, et al., 1989). Cholinergic synaptic elements e.g. ACh synthesizing and degrading enzymes, choline acetyltransferase and acetylcholinesterase are present in the CNS of insects in much higher concentrations than that in the vertebrate CNS (e.g. Prescott et al., 1977; Maxwell et al., 1978; Breer, 1981). The majority of cholinergic synapses in insect brain are nicotinic while the majority of cholinergic synapses in vertebrate brain are muscarinic

(Haim et al., 1979; Dudai, 1978; Breer, 1981; Ben-Barak et al, 1979; Lummis et al, 1985). Thus insects provide a relatively rich source for neuronal nAChRs. Also, in insects, the cholinergic synapses are confined to the central nervous system while the neurotransmitter at the neuromuscular junction is not ACh but glutamate (Ikeda, 1980; Jan et al,1976, Callec, 1985). This property further simplifies the system as a homogeneous source of the neuronal nAChR. Furthermore, there is good evidence that α-Bgt binds to a physiologically functional nAChR of the insect nervous system (for review see Breer and Sattelle, 1987). In the cockroach Periplaneta americana, iontophoretically applied acetylcholine to the sixth abdominal ganglion results in a depolarization of the giant interneuron 2 at normal resting potentials. This action could be mimicked by nicotinic agonists and blocked by nicotinic antagonists and α-Bgt. The result suggested the nicotinic cholinergic nature of the receptor and that α-bgt indeed bound to a physiologically functional receptor in cockroach. In the migratory locust, an α-Bgt affinity-purified protein complex from CNS showed 250 kD but only one band with apparent mw of 65 kD was identified on SDS polyacrylamide gels after silver staining. The protein complex was shown to form functional ACh-gated channels when reconstituted in planar lipid bilayers (Breer ,1985; Hanke and Breer et al., 1986). Although there are some questions about the subunit composition of this receptor, these experiments demonstrated that the α-Bgt binding protein is a

nAChR by nature. The availability of α-Bgt as probe and the rich source of nAChR have made insects a good material to study neuronal nAChRs.

We have chosen Drosophila as our experimental system since this insect has the further advantage of a wealth of genetic and molecular biological tools for the analysis of structural and functional properties of the neuronal nAChR not only at a molecular level but also in vivo. Questions about the regulatory mechanisms of the receptor during early development and in the aging process can be explored in this system. These studies might provide insight into the molecular basis for the developmental disabilities in the neuronal system of humans such as Alzheimer's disease which is associated with a decrease in nAChRs (Shimohoma et al., 1986; Giacobini et al, 1988).

To understand the developmental regulation of nAChR, the first step is to define the molecular structure and function of the nAChR in adult flies. High levels of α-Bgt binding to neuropile regions of the CNS have been demonstrated (Dudai and Amsterdam, 1977; Schmidt-Nielsen et al., 1977; Rudloff, 1978). The binding protein shows strong nicotinic pharmacology and suggests that these binding sites are putative nAChRs (Gepner,1979). What are the subunit composition, subunit sizes, subunit stoichiometry, precise sites for ligand binding, carbohydrate structure and the biological and biophysical properties of the nAChR? What is its genetic organization? Those questions remain unknown. One approach to study these questions relies entirely on the

use of molecular biological techniques. The concept of "Common function , common structure" has been applied to screen cDNAs libraries using probes available from other species. However, structural and functional information about the receptor protein itself cannot be obtained through this method , especially if the receptor protein is composed of multiple subunits. Another approach starts with biochemical purification of the receptor which could provide essential information about biological activity, overall structure and properties of the protein under investigation. This information can be used as a basic standard of comparison after the gene has been cloned and expressed in a functional assay. My thesis work focused mainly on the affinity chromatography purification and biochemical, biophysical and pharmacological characterization of a neuronal nAChR from Drosophila. In addition, several internal amino acid sequences were obtained from the purified receptor and oligonucleotide probes were synthesized based on these information. The oligonucleotide probes were used for screening a cDNA library from Drosophila which allowed of the cloning and characterizing several cDNA fragments.

MATERIALS AND METHODS

Living material

The wild-type Oregon R strain of *Drosophila melanogaster* was used in all experiments (Carolina Biological Supply Co.). Flies were raised on a standard cornmeal agar medium (Lewis, 1960) in polypropylene boxes at 23° ± 2°C and 65% relative humidity. Flies were harvested in a cold room (4°C) and stored frozen at -75°C.

Chemicals

Protease inhibitors: pepstatin A, leupeptin and antipain were from Bachem; benzamidine hydrochloride, bacitracin, iodoacetamide, phenylmethylsulfonyl fluoride, aprotinin, soybean trypsin inhibitor, ovomucoid trypsin inhibitor were from Sigma.

Ligands for pharmacological studies: carbamylcholine, nicotine-tartrate, d-tubocurarine, atropine sulfate and acetylcholine bromide were from Sigma.

Toxins: α-bungarotoxin and *Naja naja siamensis* crude venom were from Miami Serpentarium Laboratories.

Methyl-α-D-mannopyranoside was from Pfanstiehl Laboratories. Na-^{125}I was from New England Nuclear, Boston. Lentils were from local supermarket (brand: Jack the Rabbit). Triton X-100 was from Amersham. Deoxycholic acid was from Sigma.

Commonly used solutions

The solutions listed in Table 1 are referred to in the text by their names (for example, "Assay Buffer").

TABLE 1. Commonly Used Solutions

Name	Composition
Assay Buffer	10 mM NaP_i (pH 7.3) 50 mM NaCl 3 mM NaN_3 1% Triton X-100
Washing Buffer	10 mM NaP_i (pH 7.3) 3 mM NaN_3 0.1% Triton X-100
[^{125}I]-α-Bgt Dilution Buffer	1 x Assay Buffer 0.06% cytocrome C
Homogenization Buffer	10 mM NaP_i (pH 7.3) 1% (w/v) sucrose 5 mM EDTA 5 mM EGTA 3 mM NaN_3
Solubilization Buffer	10 mM Tris-HCl (pH 8.5) 1.8% (w/v) Triton X-100 0.6% (w/v) sodium deoxycholate 0.2 M NaCl 10% (v/v) glycerol 3 mM NaN_3 1 mM EGTA 1 mM EDTA
Dialysis Buffer I	10 mM NaPi (pH 7.3) 1 mM EDTA 3 mM NaN_3

Name	Composition
Dialysis Buffer II	100 mM NaCl 10 mM NaP_i (pH 7.3) 1 mM EDTA 3 mM NaN_3
Column Buffer 1	10 mM NaP_i (pH 7.3) 1 M NaCl 1% Triton X-100 3 mM NaN_3 1 mM EDTA 1 mM EGTA
Column Buffer 2	10 mM NaP_i (pH 7.3) 100 mM NaCl 1 mM EDTA 1 mM EGTA 1% Triton X-100 3 mM NaN_3
Column Buffer 3	50 mM Tris-Cl (pH 8.5) 0.1% deoxycholate 1 M NaCl 3 mM NaN_3 1 mM EDTA
Column Buffer 4	50 mM Tris-HCl (pH 8.5) 0.1% deoxycholate 100 mM NaCl 3 mM NaN_3 1 mM EDTA (filtered though a 0.45 um filter)
Storage Buffer (for Lentil Lectin column)	50 mM Tris-Cl (pH 7.3) 1 mM $MnCl_2$ 1 mM $CaCl_2$ 1% Triton X-100 2% methyl α-D- mannopyranoside 3 mM NaN_3

Name	Composition
Protease Inhibitor Mixture I (PIM I)	(Mix before use) Aprotinin 20 KIU/ml Antipain 1 ug/ml Bacitracin 100 ug/ml Leupeptin 2 ug/ml Pepstatin A (in ethanol) 2 ug/ml Soybean trypsin inhibitor 5 mg/ml Trypsin inhibitor 1 ug/ml Benzamidine dihydrocholoride (in ethanol) 5 mM EDTA 1 mM EGTA 1 mM $Na_2S_2O_5$ 1 mM 6-amino-n-hexanoic acid 10 mM (Add PMSF and IAA from stock immediately before use) PMSF 10 mM IAA 10 mM
Protease Inhibitor Mixture II (PIM II)	As PIM I without aprotinin and IAA
Protease Inhibitor Mixture III (PIM III)	Benzamidine dihydrocholoride (in ethanol) 5 mM EDTA 5 mM EGTA 5 mM $Na_2S_2O_5$ 1 mM 6-amino-n-hexanoic acid 10 mM PMSF 1 mM

Preparation of [^{125}I]-α-bungarotoxin

α-Bungarotoxin (α-Bgt) was iodinated as described by Schmidt-Nielsen et al. (1977). The radioactivity was determined with a LKB γ-counter and protein was determined by micro-Lowry assay (Lowry et al, 1951) using BSA as standard protein. The differences between the absorption value of α-Bgt and BSA were corrected by comparing standard curves using pure proteins. The initial specific activity of the mixture of monoiodinated and diiodinated α-Bgt was in the range of 40-80 Ci/mmol. Monoiodinated α-Bgt was prepared by chromatography on CM-Sephadex essentially as described by Blanchard et al. (1979). The specific activity was adjusted by addition of α-Bgt to a range of 60-250 Ci/mmol and stored frozen at -70°C in [^{125}I]-α-Bgt Dilution Buffer. The [^{125}I]-α-Bgt was diluted to 30 nM before use with the same buffer.

DEAE filter binding assay

α-Bgt binding activity was assayed by incubating the sample with 5 nM [^{125}I]-α-Bgt in a final volume of 0.3 ml of Assay Buffer for 60 min at 23°C. At the end of the incubation period, the assay mixture was collected on a double layer of DEAE-filter disk which was prewetted with washing buffer in a filtration manifold apparatus (Earle Sandbek, Airville, PA). The flow rate of filtration was adjusted to about 4 ml per minute. The filter disks were washed twice with 4 ml each of Washing Buffer, dried and counted in a LKB γ-counter. Non-specific binding was determined by preincubating samples in the presence of

d-tubocurarine at a final concentration of 10^{-4} M for 20 minutes.

Preparation of Affinity Resins

α-Cobratoxin (α-Cbt) was purified from the crude venom of Naja naja siamensis as described by Cooper et al. (1972). The purity was assessed by electrophoresis in a 15% SDS polyacrylamide gel and by amino acid sequencing using 15 degradation cycles on an Applied Biosystems 477A protein sequencer. α-Cbt was coupled to Sepharose 4B (2 ml gel/mg of toxin) essentially as described by March et al. (1980). The coupling conditions were adjusted to obtain a capacity of about 35-50 pmoles [^{125}I]-α-Bgt binding sites per ml of gel.

Lentil lectin was purified from Lens culinaris essentially as described by Howard (1971) and the purity was analyzed by 10% SDS-polyacrylamide gel electrophoresis. The coupling to Sepharose 4B was carried out as described above at 1.8 ml of gel/mg of lectin. The capacity of the synthesized affinity resin was about 35 pmoles [^{125}I]-α-Bgt binding sites per ml of gel.

Preparation of solubilized receptor for purification

Unless otherwise noted all steps were carried out at 0° -4°C. Heads from Drosophila were prepared as described by Schmidt-Nielsen et al. (1977) with the modification that an equal amount of powdered dry ice was included during both the disjoining and sieving steps in a stack of sieves with sizes No. 40, 25, 10 (Wire Cloth Company, Newark, N.J.) to

assure that the tissues were kept well frozen. The isolated heads were stored overnight in a freezer at -75°C to allow the dry ice to sublimate.

Membranes for receptor purification were prepared by suspending heads to 100 mg heads per ml in Homogenization Buffer. Protease Inhibitor Mixture 1 (PIM I) was included in all steps of membrane preparation. The heads were homogenized in an ice-cooled VirTis-23 homogenizer using 6 bursts of one minute each which consists of 15 seconds of fast stirring at a setting of 90 followed by 45 seconds of slow stirring at a setting of 10. Typically, 25 g heads were homogenized in 250 ml buffer in a 500 ml VirTis glass vessel. The homogenate was centrifuged for 10 min at 2,000 rpm in a GSA rotor (Sorvall) and the supernatant was filtered through a layer of cheesecloth. The pellet was resuspended at a concentration equivalent to 100 mg heads per ml in Homogenization Buffer, homogenized and centrifuged again as described above. The two supernatants were combined and diluted by 1 volume of the Homogenization Buffer and then centrifuged for 40 min at 17,000xg (GSA rotor). The upper layer of the pellet which was whitish glossy was collected and resuspended to yield a concentration equivalent of 160 mg heads per ml in Homogenization Buffer and homogenized in a Teflon pestle-glass homogenizer using 30 strokes at 1700 rpm at 0°C. This fraction was termed the "membrane preparation" which was used for subsequent solubilization. If not used immediately , the membrane preparation was stored frozen in 50 ml plastic tubes in

liquid nitrogen.

The membranes were solubilized by adding an equal volume of Solubilization Buffer containing PIM I. The suspension was homogenized for 30 strokes at 1700 rpm in a Teflon pestle-glass homogenizer and centrifuged for 30 min at 235,000 x g (Type Ti45 rotor, Beckman). The supernatant was termed the "solubilized receptor preparation".

Affinity chromatography

The solubilized receptor preparation was dialyzed in Spectrapor 2 tubing for 15-20 minutes against 5 liters of Dialysis Buffer I until the conductivity was equivalent to a 100 mM NaCl solution. The dialyzed preparation was then ready for affinity chromatography.

Two affinity columns were used. The first was α-Cbt-Sepharose 4B column (30 ml of resin in a 60 ml plastic syringe). The column was preincubated with 30 ml of Column Buffer I containing 1 M carbamylcholine for at least one hour and washed thoroughly by one liter of Column Buffer 2. Protease Inhibitor Mixture II (PIM II) was included in the last 30 ml of washing and in each step of the later operation on the first column. The dialyzed solubilized preparation was then applied to the column with a flowrate about 150 ml per hour. The column was washed with 120 ml Column Buffer 2 and the receptor was eluted from the column by incubating the column with 60 ml of Column Buffer 2 containing 200 mM carbamylcholine for one hour. This fraction was termed "first column eluate" (1E). A 0.5 ml

aliquot was dialysed against two changes of 2 liter Dialysis Buffer II for assaying the specific activity while the rest of the sample was immediately subjected to chromatography on the second affinity column. The cobratoxin column was regenerated by incubating in Column Buffer 1 containing 1 M carbamylcholine for 2 hours followed by washing with 120 ml of Column Buffer 2 without the protease inhibitors and stored at 4°C for reuse.

The second affinity column was a lentil lectin-Sepharose 4B column. The lentil lectin column (20 ml gel bed in a 60 ml syringe) was washed with 40 ml of Column Buffer 1, 80 ml Column Buffer 2 and than 20 ml of Column Buffer 2 containing PIM II. The eluate from the α-Cbt column was applied to this column with a flow rate of about 100 ml per hour. The column was washed with 100 ml of high salt Column Buffer 3 and then washed with 40 ml of low salt Column Buffer 4, both containing PIM II and the detergent deoxycholate to replace the Triton X-100 in the previous buffer. The column was then washed with 40 ml of filtered Column Buffer 4 containing PIM 3 to replace the peptide protease inhibitors with non-peptide protease inhibitors. The receptor was eluted from the column by incubating the column for 30 minutes with 40 ml filtered Column Buffer 3 containing 10 % methyl-α-D-mannopyranoside and PIM III. The buffers at this step must be filtered to prevent the blockage of YM-100 membrane filters in the subsequent concentration step. This eluted fraction was termed "second column eluate" (2E) and was stored in liquid nitrogen. A 0.5

ml aliquot was dialyzed against two changes of 2 liter of Dialysation Buffer II for assaying the specific activity. The lentil lectin column was regenerated by incubating 1 hour with 20 ml Column Buffer 1 containing 20% methyl-α-D-mannopyranoside followed by washing with 60 ml of Column Storage Buffer. The column was stored at 4°C for reuse.

Photoaffinity labeling

The photosensitive hetero-bifunctional crosslinker methyl-4 azidobenzoimidate was sythesized as described by Ji (1977).

The derivatizing of the synthesized crosslinker to monoiodo-125 I-α-bungarotoxin was as described by Ji (1977) with the following modifications. A solution containing 200-400 nM monoiodo-125 I-α-bungarotoxin, 60 mM sodium chloride and 3.3 mM sodium phosphate buffer pH 7.3 was adjusted to pH 9.5 by the addition of 20 mg sodium carbonate and 25 mg sodium bicarbonate. All subsequent steps were conducted in the dark using a Wratten #2 safe light. At 0, 2, 4, and 7 h, 1 mg of synthesized methyl 4-azidobenzo-imidate was added to the toxin and the mixture was stirred continuously at 4°C for a total of 22 h. Cytochrome c (1mg) was then added as a carrier and the mixture was purified on a Sephadex G-25 column (0.7 x 30 cm) which was equilibrated and eluted with 3.3 mM NaP_i buffer, pH 7.3. 0.5ml fractions were collected and the first major peak fractions were pooled and stored in the dark at 4°C in the presence of

0.02% sodium azide.

Photoaffinity labeling of the derivatized α-bungarotoxin to receptor was carried out as described by Ji (1977, 1979). The labeling mixture was equilibrated in the dark for 40 minutes before being radiated under UV light for 40 minutes. Controls were preincubated with 10^{-5} M α-Bgt.

Sucrose Density Gradient Centrifugation

Linear 5% - 20% (w/v) sucrose gradients were prepared in H_2O containing 10 mM NaP_i, pH 7.3, 500 mM NaCl, 1% Triton X-100, 1 mM EDTA, 3 mM NaN_3. The sucrose solution was autoclaved prior to preparation of the gradient. 200 ul of sample containing protease inhibitors was loaded onto a 4.8 ml of gradient and centrifugation was carried out as described by Martin and Ames (1961), using a Beckman SW 50.1 rotor, at 45 K for 12 hours. 150 ul fractions were collected from the bottom of the tube. For the determination of the effect of bound detergent on the sedimentation coefficient, a parallel gradient was run in which D_2O was used to replace the H_2O in the above buffer. The standards used were E.coli ß-galactosidase ($S_{20,w}$ = 15.9), bovine liver catalase ($S_{20,w}$ = 11.3) and yeast alcohol dehydrogenase ($S_{20,w}$ = 7.6) (all from Sigma, and assayed according to Sigma directions).

Gel filtration

Gel filtration was carried out using a siliconized Sepharose 4B column (50cm x 1cm) in the same buffer used for sucrose density gradient centrifugation. The protein

markers used and their Stokes radii were : thyroglobulin (8.5nm), ß-galactosidase (6.9nm), ferritin (6.1nm), catalase (5.2nm) and alcohol dehydrogenase (4.5 nm) (obtained from Sigma and assayed according to Sigma directions).

Protein Determination

The standard procedure of a modified Lowry assay (Peterson, 1983) was used for samples containing 5-100 ug protein in a volume of 1 ml. For samples containing 1-20 ug protein in a volume of 0.5 ml, the microassay procedure was used (peterson, 1983).

Amino Acid Analysis

Amino acid analysis was carried out according to Henrickson and Meredith (1984). The PTC amino acids were separated on a C-18 column using an Applied Biosystem 130A Microbore HPLC.

Protein concentration

Large volumes of sample were consecutively concentrated in Amicon 8050 and Amicon 8010 concentrators with Diaflo Ultrafiltration membrane YM100 (Amicon Div., W. R. Grace & Co. MA.) under a pressure of 20 psi argon on ice with constant stirring. The eluate of the lentil lectin column was concentrated by this method to about 1-2 ml with a flow rate of about 2 ml per minute.

Proteins in small volumes (less than 2 ml) were precipitated with methanol and chloroform as described by

Wessel et al (1984) .

SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis was carried using 0.5 mm thick, 8 cm x 10 cm SDS polyacrylamide minigel (10%). The procedure of Laemmli (1970) for the treatment of sample was modified such that all receptor samples were loaded without heating. For the purpose of amino acid sequencing, the gel was polymerized overnight before use (Hunkapiller, 1983). The prestained molecular weight markers used were : α_2-macroglobulin (M_r = 180,000), ß-galactosidase (M_r = 116,000), fructose-6-phosphate kinase (M_r = 84,000), pyruvate kinase (M_r = 58,000) fumarase (M_r = 48,500), lactic dehydrogenase (M_r = 36,500) and triosephosphate isomerase (M_r = 26,600) (from Sigma).

Autoradiography

SDS polyacrylamide gels were soaked for 2 hours in 0.1% (v/v) glycerol in 10% (v/v) acetic acid, dried on filter paper in a gel dryer and exposed for autoradiography using Kodak XAR film with a Cronex intensifying screen (DuPont Co., Delaware).

Electroblotting

Proteins separated by SDS PAGE were electroblotted in a Bio-Rad transblot system onto two layers of nitrocellulose filters (Micron Separation Inc. Ma.) as described by Towbin (1979) with modifications of blotting conditions. A constant

current of 20 mA , 16-20 hours was used to blot a 0.5 mm thick gel. After transferring, polypeptides were stained for 1 minute with 0.1% Ponceau S dye in 1% aqueous acetic acid. Excess stain was removed from the blot by a brief wash in 1% aqueous acetic acid. Protein containing regions of the filter were cut out and same bands were pooled in one Eppendorf tube and destained by briefly in 200 uM NaOH. The filters were than washed with distilled water and stored wet in -20 °C (Aebersold, 1987).

In situ tryptic cleavage of electroblotted proteins

The protocol was as described by Aebersold (1987) with the following modifications. The destained filters were preincubated with 1.2 ml of 0.5% PVP-40 in 100 mM acetic acid for 30 minutes at 37°C in order to prevent absorption of trypsin to the filters during the digestion and than washed thoroughly with water. The filters were cut into small pieces of about 1 x 1 mm on a teflon block and put into a siliconized glass tube (0.4 mm x 4.0 mm). 30 ul of trypsin digestion buffer (100 mM Tris-HCl, pH 8.2/acetonitrile, 95:5 (v/v) with 0.2% Ca^{++} was added. Trypsin (from Boeringer Mannheim) was freshly prepared 1 mg/ml in 0.1% trifluroacetic acid (TFA) and added to the reaction in an enzyme-to-substrate ratio of 1:20 (w/w). For control, blank filters were digested with the same amount of trypsin. The mixture was digested overnight and then the whole reaction was frozen at -20°C or loaded onto the HPLC after acidification.

Reverse phase HPLC of the cleavage fragments

Tryptic digested filters were acidified with 5 ul of 10% trifluoroaccetic acid and sonicated for 30 seconds. The filters were then spun down at 2,000 rpm for 2 minutes and the supernatant was saved in a fresh siliconized glass tube. The filters were reextracted with 15 ul of HPLC column buffer A (see below), sonicated and spun as above. The supernatants were pooled and spun once more. The supernatant was taken up into a 50 ul Hamilton syringe without touching any particles at the bottom which would block the column and loaded immediately onto the HPLC column. The tryptic fragments were separated by a narrow-bore reverse phase HPLC system using a dual syringe Brownlee micropump. The analytical column used was a Brownlee Aquapore RP-300, 2.1x100 mm (or 1.0 x 100 mm, see text). The following buffer system was used . Buffer A: 0.1% TFA (Sequencing grade, Pierce) in water (Pierce). Buffer B: 0.08-0.095% trifluroacetic acid in acetonitrile/ H_2O, 70:30 (v/v). The gradients used were 0-100% B or 10-60% B as mentioned in the text. All experiments were carried out with columns at 25°C, and a flow rate of 100 ul/min. Peptides were detected by simultaneous monitoring at 220 nm, with a Waters 490 detector and separation profiles were monitored by the Nelson analytical program. For 1.0 x 100 mm column, the profiles were also monitored simultaneously by a Kipp and Zonen recorder, model BD 40. In all cases, fractions were collected manually.

Peptide sequencing analysis

RP-HPLC separated peptide fractions were sequenced by loading onto a glass filter directly or by lyophilizing without thawing the protein sample and then loading, using an Applied Biosystems model 477A automated sequencer, operated according to the manufacturer's instructions. Stepwise liberated PTH-amino acids were identified using an on-line 120A PLC system equipped with a PTH C18 column (Applied Biosystems) .

Oligonucleotide probe synthesis

The internal peptide sequences were backtranslated into nucleotide sequences by using GCG (The Genetics Computer Group at University of Wisconsin) backtranslation program (Devereux, et al., 1984). The codon usage parameters used was the highly expressed gene pattern in Drosophila melanogaster (Sharp, 1988). The degenerate nucleotide sequences were adjusted (see text for detail). The complimentary sequences were synthesized as oligonucleotide probes in an Applied Biosystems Model 380B DNA synthesizer according to the instructions given by the company.

Screening of the cDNA library

Drosophila whole adult cDNA library (Clontech, Inc.) was used for screening. Standard protocols as described in the manual by Sambrook et al. (1989) were used. Probes with similar melting temperature were pooled for primary screening at a temperature of 15°C below melting temperature

in 6xSSPE for 48 hours. The washing conditions used were 10°C below melting temperature in 2xSSPE for 30 minutes. In the process of rescreening, a single probe was used. The hybridization conditions were as in the primary screening with the washing temperature 5-8°C below the melting temperature.

Southern Blotting

Inserts from positive clones in the library screening were subcloned into the plasmid vector pBluescript (Stratagene Cloning System). Insert fragments were released with EcoR I and separated on an 1% agarose gel. Southern blotting was carried out and the final wash of the hybridized filter was 2xSSPE with a temperature 8°C below the melting temperature. The filters were exposed for autoradiography as in Sambrook, et al. (1989)

RESULTS AND DISCUSSION

Affinity Purification of an α-Bgt binding activity

A. Characterization of α-Bgt binding activity in membrane preparations from Drosophila heads

1. Properties of α-Bgt binding

The existence of specific α-Bgt binding components in head extracts from Drosophila was reported previously by several groups. However, there are controversies among those reports regarding the binding properties. Some groups have described the existence of a single binding site with

K_d values ranging from $2x10^{-9}$ M to 1.6 x 10^{-10} M (Schmidt-Nielsen et al., 1977; Dudai 1978; Jimenez and Rudloff, 1980), while others have suggested the existence of multiple binding sites with K_d values of $1x10^{-10}$ M for a higher affinity binding site and $4x10^{-9}$ M for a lower affinity binding site (Schloβ, 1988). Before initiating the purification process, it was necessary to verify the existence of specific binding sites in our membrane preparation.

The membranes were prepared as described in Methods. 5 ul of sample (about 0.35 mg of protein) was used for the saturation binding assay. [^{125}I]-α-Bgt concentrations in the assays ranged from 0.05 nM to 60 nM. Controls were set up at each [^{125}I]-α-Bgt concentration in which the samples were preincubated with inhibitor d-tubocurarine at 10^{-4} M for 20 minutes for the determination of non-specific binding. As shown in Fig.1, the total binding of [^{125}I]-α-Bgt to the membrane did not saturate, but when the background binding was subtracted, the net binding leveled off which showed that the binding was saturable. This property meets the basic criteria for specific binding.

The data from Fig.1 were analyzed by a Scatchard plot using a Marquardt-Levenberg non-linear regression curve fitting procedure (Meeker et al., 1986) performed by Sigmaplot (Version 4.0). The plot (Fig.2) suggests the existence of more than one binding sites with notable differences in the affinities to α-Bgt. The higher affinity binding class has K_{d1} of 4.1 x 10^{-10} M and lower affinity

binding class has K_{d2} of 3.3 x 10^{-9} M. The maximal binding sites for both high and low affinity classes were 0.367 pmol/mg protein (B_{max1}) and 0.385 pmol/mg protein (B_{max2}) with the ratio of 1:1 of high:low affinity. Our results agree with previous report (Schloβ, 1988) regarding the binding affinities (1x10^{-10} M for high affinity and 4x10^{-9} M for low affinity) but differ in the ratio of the maximal binding sites (1:4 of high : low). The values of K_d and B_{max} are summarized in Table 1.

2. Pharmacological properties

Previous reports revealed the nicotinic nature of the α-Bgt binding activity (Schmidt-Nielsen et al, 1977; Gepner, 1979). From the groups reporting two types of binding sites, data were presented indicating that both binding sites were nicotinic (Schloβ, 1988). Since we observed the existence of more than one type of binding sites, it was interesting to test the pharmacological properties of these binding sites in our preparation. Competitive binding with various cholinergic ligands was carried out. The binding reaction was one hour allowing to reach steady state for both competitive ligands and for α-Bgt.

The profile of inhibitory ranking order of ligands is summarized in Fig.3 . The ranking order was α-Bgt > nicotine > d-turbocurarine > acetylcholine > atropine. The values of the IC_{50} are listed in TABLE 3. Of the nicotinic agents, the agonist nicotine has an IC_{50} of 4.5x10^{-7} M and the antagonist d-tubocurarine has an IC_{50} of 2.6 x 10^{-6} M; atropine, a

muscarinic agent, has an IC_{50} of 5.7 x 10^{-5} M. These results demonstrate that nicotinic ligands are much better inhibitors for [^{125}I]-α-Bgt binding than muscarinic agents. This characteristic identifies the α-Bgt binding proteins in the Drosophila membrane fraction as nicotinic. In addition , the shape of inhibition curves by nicotine and d-tubocurarine are characterized by shallow gradients (Limbird, 1986). This further indicates that the membrane fraction contains more than one type of α-Bgt binding sites, and both of which were nicotinic.

3. Photoaffinity labeling

A major problem in receptor purification is the proteolytic degradation by endogenous proteases. This problem becomes more severe as the purification progresses. Since the relative concentration of the protein of interest becomes higher and higher , a trace contamination of proteases will result in serious degradation of the purified protein. Without knowing the subunit composition and molecular weight of protein of interest, proteolytic degradation will frustrate the results. Here are just two examples. First, it has been reported that receptors from muscle and electric organ are easily proteolyzed during purification to give the appearance of being composed of only α subunit rather than α, β, γ and δ subunits (Lindstrom et al., 1980). Second, the α-Bgt binding component from human muscle was purified and only a single component was identified, however, with increased experience, three and

then finally four subunits were purified (Lunt, 1986). Thus, in the whole process of our purification experiments, great precautions were taken to prevent proteolysis (see below). However, it still will be difficult to judge how well the proteolysis was controlled without an independent confirmation of the subunit composition. To monitor the effect of proteases on α-Bgt binding proteins during our purification process, photoaffinity labeling was used.

[^{125}I]-α-Bgt was derivatized with the hetero-bifunctional cross-linking agent methyl-4-azidobenzoimidate. This derivatized [^{125}I]-α-Bgt was incubated with membrane fractions and crosslinked to the α-Bgt binding proteins *in situ* in the membrane by photo-activation. The labeled membrane sample was analyzed by SDS-PAGE and followed by autoradiography.

This method allowed us to visualize the molecular weight of the labeled subunit(s) before purification which could set up a standard for comparison with the purified sample. The logical expectation is that if there is proteolytic degradation, the molecular weight of protein(s) will become smaller as the purification proceeds. On the other hand, if proteolysis is well controlled, there should be no change in the molecular weight of the labeled subunit(s) before and after purification. Another advantage of this method is that it could reveal the subunit composition before purification if all subunits could be labeled.

As shown in Figure 4, two bands were detected in the *in*

situ photoaffinity labeling experiments. After the correction for the molecular weight contribution by bound [^{125}I]-α-Bgt, the two bands were estimated to have molecular weights of 57K and 44K daltons. The specificity of the photo-affinity labeling was demonstrated by complete blockage of the labeling of these two bands by preincubation of the sample with unlabeled α-Bgt at 10^{-5} M.

Since some subunits of the binding proteins might not be photoaffinity labeled, we could not predict the subunit composition just based on the photoaffinity labeling results.

B. Design of the purification scheme

The purposes of the purification were to isolate and characterize the purified receptor and to obtain its internal amino acid sequence(s). To achieve this objective, maintaining the bio-activity of the receptor and maximizing the yield were the basic concerns in the designing of the purification scheme.

The strategy used for the purification of *Drosophila* nAChR is based on the preliminary data by Schimdt-Glenewinkel et al. (1980) and shown in Fig. 5. The scheme is composed of mainly three parts: the preparation of the sample for purification, the affinity chromatography , and the concentration step.

Great caution was taken in preventing proteolytic degradation which included the use of a mixture of protease inhibitors during the whole purification process; the

manipulation of each step at low temperature (0-4°C); the rapid processing of the tissue and the whole purification procedure; and sterilization of critical solutions.

Drosophila heads were used as starting material since this tissue contained 56% of the toxin binding activity but only 12% of the total protein found in flies (Schmidt-Nielsen et al., 1977). Non-membrane fractions were removed by differential centrifugation. The membranes were solubilized under mild condition for best yield and used for affinity chromatography.

Two affinity columns were used: an α-Cobratoxin Sepharose 4B affinity column and a lentil lectin Sepharose 4B affinity column. α-Cobratoxin had proven to be very useful in the purification of muscle type nAChR , which also have a high affinity to the α-Bgt binding proteins in Drosophila (IC_{50}, 3.3 x 10^{-8} M, Gepner, 1979). This property made it a suitable candidate as a probe for the purification of α-Bgt binding protein. However, we found that the affinity between the immobilized α-Cbt and the α-Bgt binding activity was not strong enough that a high salt (0.5 M NaCl) could wash off substantial amount of the binding activity. A lentil lectin column was used based on the glycoprotein nature of receptors. The α-Cobratoxin Sepharose 4B affinity column eluate could be immediately loaded onto the lentil lectin column without dialysis. The lentil lectin column could be washed with high salt and the eluate from the lectin column could be used directly for measuring the specific activity without dialysis. This obviated a time

consuming dialysis step which often results in the loss of binding activity.

The final concentration step was necessary for the purpose of sequencing. The Amicon concentrator was used to achieve a rapid concentration without causing the aggregation and denaturation of the purified protein . This step also provided further purification since the YM-100 membrane in the concentrator has a molecular weight cut off of 100 kD.

The purification procedure was evaluated by the change in specific activity. The binding activity was measured by DEAE filter assay. The protein amount was determined by a modified Lowry assay (Peterson, 1983). For the purified receptor, protein was determined by amino acid analysis. The subunit composition was visualized by silver stain after separation by SDS PAGE and also by photoaffinity labeling. The authenticity of the purified receptor was determined by comparing the pharmacological properties of the receptor before and after purification.

C. Preparation of the affinity columns

1. Purification and characterization of α-Cobratoxin from Naja naja siamensis venom

The first affinity column was a α-Cobratoxin-Sepharose 4B column. The α-Cobratoxin was purified from snake Naja naja siamensis venom as outlined in Fig. 6. The purity of the purified toxin was examined since trace contamination with proteases at this step will cause severe degradation of

the receptors. The purified α-Cbt was separated on a reversed phase HPLC column (RP-300) and the elution profile showed a single sharp peak (Fig. 7) . This peak fraction was collected and sequenced by gas phase amino acid sequencing. The first 15 N-terminal amino acids of the purified α-Cbx were compared with the published N-terminal sequence of the toxin (Tu, 1973) and both are exactly the same (Fig.7). We further tested the bioactivity of the purified α-Cbt by inhibition of [^{125}I]-α-Bgt binding to membrane preparations. The derived IC_{50} of α-Cbt was 4 x 10^{-8} M (Fig.8) which is in the range of reported data (Gepner, 1979).

2. Purification and characterization of lentil lectin from Lens culinaris

The lentil lectin was purified from Lens culinaris as described by Hayman and Crumpton (1972 , Fig 9). The purified sample was analyzed by 10% SDS-PAGE and visualized by silver staining (Fig. 9). A single band with the expected mw of 24.5 kD was detected (Howard, 1971).

3. Synthesis of α-Cbt-Sepharose 4B and lentil lectin-Sepharose 4B affinity columns

Both affinity columns were synthesized as described by March et al. (1980) with some modifications. The phenomenon that a high capacity affinity column (500 pmole binding sites/ml resin) results in almost 50% lower recovery than moderate capacity (around 50 pmole binding sites/ml of resin was reported previously (Gotti et al., 1982). The low local

concentration of the substituted toxin is crucial for more efficient desorption and a better recovery . While the ligand : resin ratio was kept constant, the ligand substitution degree was optimized by varying the amount of cyanogen bromide used for the activation of the resin and the time needed for cyanogen bromide activation in the coupling step. The capacities of the synthesized resins were tested. The final conditions adopted were 40 mg cyanogen bromide/ml gel and 12 hours of coupling with approximately 80% coupling of the ligand. The capacities of the two columns synthesized were both in a range of 30-35 pmol binding sites/ml of the resins.

D. Preparation of solubilized extract for purification

1. Solubilization of α-Bgt binding proteins from membrane extract

Purification started from the preparation of membrane from Drosophila heads. The effect of pH on solubilization was first tested. Fly heads were homogenized in buffers which differed from each other in pH. Following solubilization, preparations were assayed for the recovery of binding activity. The results standardized by the binding value from pH 7.5 buffer show that there were no obvious differences in the recovery in the pH values ranging from pH 4.0-10.5 (Fig. 10). The dramatic decrease of binding activities at pH 2.5 and pH 3.0 might be due to the inactivation of binding protein in such an acidic environment.

Membrane preparations were prepared from about 25 g of fly heads per batch as described in Methods and frozen immediately in liquid nitrogen. The frozen samples could be stored at -70°C for days without loss of binding activity and several batches were pooled for solubilization.

A low yield in solubilizing Drosophila α-Bgt binding proteins was reported previously by Dudai (1978) and Gepner (1979) and the reasons are still not known. Gepner (1979) made a systematic evaluation of numerous detergents and salt concentrations and the best yield of 25% solubilization was reported by using buffer containing high salt (0.5 M NaCl) and 1% Triton X-100, pH 7.3. This protocol was first tried and the yield was in the same range as reported by the author. However, the high salt concentration in the solubilized sample caused low adsorption on the α-Cbt affinity column. Thus the salt concentration in the solubilized sample had to be lowered from 0.5 M to 0.1 M NaCl. The dialysis step took one hour and resulted in a decrease in the binding activity by 10-15%. If the dilution method was adopted, the time for chromatography was greatly lengthened which also caused loss of activity. Another solubilization condition was described using a mixed detergents of 1.8% Triton X-100 and 0.6% sodium deoxycholate at a salt concentration of 0.2 M at pH 8.5 (Schloβ et al., 1988). Using this procedure, as with the earlier method, approximately 25% of the binding activity was solubilized. However, the salt concentration could be brought down from 0.2 M to 0.1 M in only 15 minute by dialysis without change

of the dialysis buffer. The loss of activity in this step was only about 2%. The time factor favored this protocol which was adopted in our subsequent experiments for solubilization. The overall recovery of the binding activity after dialysis was about 20% of the membrane fraction.

2. Characterization of the solubilized preparation

Scatchard plot analysis of the binding data was carried out for the solubilized α-Bgt binding proteins. The question was whether both types of binding sites were equally soluble or if there was differential solubility between the two classes of binding sites. The amount of protein used in the saturation binding of [^{125}I]-α-Bgt to the solubilized preparation was about 39 ug. The results (Fig. 11) showed a non-linear Scatchard plot with K_{d1}, 3.8 x 10^{-10} M and K_{d2}, 2.9 x 10^{-9} M which were essentially unchanged from the crude membrane preparation (Table 2). However, the ratio of B_{max1} and B_{max2} was changed from a ratio of 1:1 in the membrane preparation to 1:2 in the solubilized preparation (Table 2) which suggests that the higher affinity binding site might be more difficult to solubilize than the lower affinity binding site. This observation was in general agreement with the report about the two types of α-Bgt binding sites in Drosophila that the ratio of B_{max1} : B_{max2} was shifted from 1:4 in the membrane preparation to 1:10 in the solubilized preparation (see data in Schloβ, 1988). This phenomenon suggests that the physical environments of the two types of binding proteins in the membrane might be different.

E. Affinity purification of α-Bgt binding proteins

1. Monitoring the purification process

The purification process was monitored by the specific activity of major fractions. The binding activity was determined by the DEAE filter assay method while the mass of protein was determined by different methods. For most samples from the purification, total protein amount was measured by the standard assay procedure of a modified Lowry assay (Peterson, 1983). The advantage of this method is that it allows for the determination of 5-100 ug/ml of proteins in the presence of detergents. However, we found that the protein values determined were considerably influenced by factors such as EDTA and EGTA which were included in the purification process as protease inhibitors at 1 mM concentration. All samples used for this assay were dialyzed thoroughly against Dialysis Buffer II before being assayed. The limitation of the Lowry assay (also the modified method by Peterson, 1983), made it necessary that purified receptor samples were subjected to amino acid analysis for accurate measurement.

The protein composition of the samples at each purification step was also monitored by SDS-PAGE following the Laemmli protocol. To our surprise, severe aggregation was observed as more than 50% of the proteins were trapped in the stacking gel. Since the mechanisms causing protein aggregation are not well understood, several routinely used methods for preventing aggregation were tried. 8 M urea was added to the sample before loaded on the gel and the gel

still showed large portions of the protein in stacking gel. An hydroxylapatite column was used to replace the mixture of detergents (Triton X-100 and deoxycholate in the sample) with only deoxycholate since detergent mixture might influence the function of SDS during PAGE. However, the result still was the same aggregation. We realized that since the samples still had bioactivity before being denatured by loading buffer that the aggregation must occur after the loading buffer being added. The sample treatment condition might also have some effect on the aggregation. The heat treatment step was tested using the following conditions: 100°C, 3 minutes boiling; 65°C 10 minutes or direct loading without heating. A stunning difference was observed which is shown in Fig. 12 and 13. Heating for 3 minutes in boiling water caused dramatic aggregation of the purified proteins such that all proteins stuck in the stacking gel layer of the gel (Fig 12. S) and the same was seen for heating at 65°C for 10 minutes (data not shown). On the contrary, direct loading of the sample without heating prevented aggregation and produced well separated bands on the gel. Heat resulted aggregation also observed in the receptor proteins in the membrane (Fig. 13). The membrane sample was photo-affinity labeled. Samples were then spun for 2 minutes in a bench top microfuge at full speed either after heating or without heating and the supernatants were loaded on the gel. The effect of heating on aggregation was clearly visible. In heated samples, no clear bands were seen while in non-heated samples, there was a reasonable

separation. The effect of low temperature on aggregation was also tested. Protein samples were stored in -70°C overnight and allowed to thaw at room temperature for 10 minutes before loading on the gel without heating. Good separation could be achieved (data not shown). Therefore the no-heating-at-all protocol was adopted in all SDS-PAGE experiments.

2. Affinity Chromatography

The solubilized α-Bgt binding proteins were dialyzed and affinity purified by α-Cbt-Sepharose 4B affinity column and lentil lectin-Sepharose 4B affinity column as outlined in Fig. 5. Both resins had a capacity of about 30-35 pmol/ml and 50% of the loaded activities were recovered from each column. The mixture of detergents Triton X-100 and deoxycholate was replaced by deoxycholate in the lentil lectin column by thorough washing before the receptors were eluted. The eluate from the second affinity column was concentrated in an Amicon cell using an YM-100 membrane.

The method described here allowed for the purification of a relatively large amount of the α-Bgt binding protein i.e. in the nanomole range which is sufficient for characterization of biochemical and physical properties and internal amino acid sequence analysis. A typical purification process is summarized in Table 4. The overall recovery was about 2% of the starting activity. The specific activity of the Amicon YM100 concentrated sample was about 3.9 umol/g protein. The molecular weight of the receptor

protein was calculated to be 270,000 (see result below). If each protein contains one binding site, then the theoretical specific activity for the pure sample should be 3.7 umol/g protein. If the receptor protein contained two binding sites, the theoretical specific activity would be 7.4 umol/g protein. Assuming there are two binding sites per protein as in muscle type nAChR, our sample was about 53% of the theoretical purity assuming no loss of binding activity during the purification. As compared with the starting specific activity of the homogenate (0.67 umol/mg protein), an overall purification of about 5820 fold was achieved.

A silver stained SDS-PAGE gel containing major fractions of the purification process is shown in Fig.14. The same amount of protein (10 ug) was loaded for samples H (homogenate); M (membrane); S (solubilized extract); 1E (first column eluate). For 2E (concentrated preparation of second column eluate), 5 pmoles of binding sites were loaded. The protein pattern changed considerably after the α-Cbt affinity column. However, this column had the limitation that high salt wash resulted in the loss of binding activity in the washing step. A gradient salt wash from 200 mM to 600 mM would cause a loss of 40-60% of the bound activity. This problem was overcome by the lentil lectin affinity column which could be washed by 1 M salt without obvious loss of binding activity. Two major bands appeared in the lentil lectin affinity column eluate after YM 100 membrane concentration with molecular weights of 44 KD and 57 KD.

Characterization of the purified receptors

A Subunit Composition

1.Sucrose density gradient analysis

If the two major protein bands indeed are the components of the receptor protein, they must be associated with the binding activity. The purified sample was subjected to a continuous sucrose density gradient centrifugation (5-(5-20%) in the presence of protease inhibitors. Fractions of a size of 110 ul were collected and the profile of α-Bgt binding activity of the fractions was determined as shown in Fig.15. The binding activity appeared as a single sharp peak. Since the amount of protein in each fraction was rather low, fractions were pooled as shown in the figure and analyzed by SDS-PAGE. The silver stained gel clearly showed two protein bands with molecular weights of 44 KD and 57 KD (lane 2) which strongly indicate that these two protein bands correspond to the α-Bgt binding activity. Unfortunately, the specific activity of this pooled peak fraction could not be determined due to the small amount of the protein.

A weak band of higher molecular weight (60 kD) was also observed in lanes 2 and 3. It is unlikely to be a component of the receptor since the intensity of the band was not correlated to the binding activity : the binding activities loaded in lane 2 was 4.6 pmole and 1.5 pmole in lane 3 while the intensity of this 60 KD band in lane 3 was much higher than in lane 2.

Autoclaving of the sucrose immediately before preparing the gradient was found a necessity to prevent protein degradation (data not shown).

2. Photoaffinity labeling

To examine if these two protein bands are indeed receptor subunits which bind α-Bgt, the purified sample was labeled by photoaffinity labeling. This should be the most direct evidence for the relationship between the polypeptide bands and the binding protein.

The result of photoaffinity labeling of the purified receptor is shown in Fig 16. The pooled peak fraction from the sucrose gradient (pool 2, Fig 15) was labeled and both bands (57 kD and 44 kD) were clearly labeled (lane 5, in Fig 16). Taken together with the result in lane 2, Fig. 15, these two protein bands correspond to the binding activity and both could be photoaffinity labeled. This evidence strongly suggests that the two bands are receptor subunits both would bind to α-Bgt or in the vicinity of the α-Bgt binding site.

Controls were set up in two groups. Samples in group one were photoaffinity labeled in situ in membrane and the subjected to different manipulations. This group of samples was used to monitor the proteolysis: membrane preparation labeled in situ (lane 1); in situ labeled membrane preparation solubilized and sedimented through a sucrose gradient (lane 3.). The other group of samples was purified first and than photoaffinity labeled and this group

was used to verify the composition of the purified receptor. The purified sample (concentrated lentil lectin column eluate , lane 4) was labeled. The result of all these experiments agree with each other and revealed the existence of two protein bands with molecular weights of 57 kD and 44 kD.

The consistency of the results indicated that proteolytic degradation appeared to be reasonably well controlled in the whole purification process since no changes of molecular weight were observed. The fact that the purified proteins could be photoaffinity labeled showed that the native conformation of the receptor proteins remained intact even after about 6000 fold purification and that the receptor is composed of two types of protein subunits both of which could be photoaffinity labeled.

Although the photoaffinity labelling experiments seem to exclude the possibility of proteolytic degradation, the possibility of co-purification of peptides with the similar molecular weight as reported in the case of GABA receptor purification by Seeberg's group (Levitan et al., 1988) still exists.

Breer et al (Breer, 1986; Hanker and Breer et al., 1986) reported that the reconstitution of purified α-Bgt binding protein from locust ganglia (250 kD) in planar lipid bilayers generated functional acetylcholine-gated channels. The SDS polyacrylamide gel analysis showed that the purified protein was composed of a single type of polypeptide of 65 kD. Since muscle type nicotine AChRs are composed of four

types of highly homologous subunits which might be due to the duplication of one ancestral gene, the author suggested that this insect nAChR may correspond to an ancestral receptor which is composed of ligand-binding α-subunits only. Because receptors are easily subjected to proteolytic degradation(e.g. Lunt, 1986), Breer's results should be viewed with some caution. Our results clearly revealed the existence of at least two types of subunits.

B. Biophysical properties

To obtain the molecular weight of the purified receptor, the Stoke's radius was first determined by gel filtration. The α-Bgt binding protein was eluted in a sharp symmetrical peak and the Stoke's radius was determined to be 7.4 nm (Fig.17).

The apparent sedimentation coefficient at 20°C, in H_2O was determined to be 9.5 by sucrose density gradient centrifugation (Fig. 18). Since the α-Bgt binding protein exhibited properties of an integral membrane protein, most likely, large amounts of the detergent would bind to it during solubilization as has been reported for other receptors (Meunier et al. 1972, Reynolds and Tanford, 1976, Siegel et al. 1981). The contribution of detergent to the mass of the α-Bgt binding protein can be determined by sedimentation analysis in H_2O and D_2O in the presence of suitable marker proteins which do not bind detergent (Meunier et al., 1972, Clarke, 1975). As Fig. 18 shows, the apparent sedimentation coefficient of the purified α-Bgt

binding protein was 9.5 in H_2O but only 8.2 in D_2O. This indicates the presence of tightly bound detergent in the purified receptor.

The partial specific volume v of the receptor-detergent complex was calculated to be 0.768 ml/g (as Clarke, 1975) using sedimentation coefficient values obtained in H_2O and D_2O. The value is considerably higher than that of the average soluble protein: v = 0.73 ml/g (Salvaterra and Mahler, 1976).

Based on the values of Stoke's radius, $S_{20,w}$ and the partial specific volume , the molecular weight of receptor-detergent complex was calculated to be 325,729 (Clarke , 1975). The contribution of detergent to the molecular weight was calculated to be about 20% which results in a receptor molecular weight of about 270,000.

The frictional ratio of the receptor-detergent complex was calculated to be 1.72 (Clarke, 1975) which indicated a highly asymmetric protein complex this is compatible with the expectations of the properties of transmembrane proteins.

C. Pharmacological properties

1. Pharmacological specificity

The pharmacological properties of the purified receptor were investigated. Competitive binding studies showed that the ranking order of cholinergic ligands binding to the purified receptor was nicotine > d-tubocurarine > acetylcholine > atropine which was same as the ranking order

before purification (Fig.19). The values of IC_{50} of various cholinergic ligands were in the same range as the values before purification (Table 2). Thus, the pharmacological properties of the α-Bgt binding proteins appeared to be preserved during the purification process.

2. Binding properties

Scatchard plot analysis was carried out to see whether the purification process would separate the two classes of the binding sites. Interestingly enough, the Scatchard plots still showed that there was more than one binding site in the purified sample with K_{d1} $3.1x10^{-10}$ M and K_{d2} $2.3x10^{-9}$ M (Fig.20). These values were in the same range as before purification . However, the ratio between B_{max1} and B_{max2} was changed from 1:1 in membrane to 1:2 in the solubilized preparation and 1:3 in the purified sample (Table 2). These results suggested that the experimental conditions used were not sufficient to physically separate the two types of binding sites even after 5820 fold of purification. However the changes in the ratio of B_{max} implicated the physical differences between the two types of binding sites.

D. Summary of the receptor properties

The biophysical and molecular properties of the purified receptor are summarized in Table 5. Since the two subunit peptides have molecular weights of 44 kD and 57 kD respectively, the 270 kD binding activity must be composed of multiple subunits. However, the stoichiometry of the

receptor subunits in the receptor is not known yet.

Internal Amino Acid Sequencing

A. Electroblotting

1. Protein concentration

To obtain enough protein for sequencing, the amount of protein in each band in the gel should be as high as possible. The sample after Amicon concentration needed still to be further concentrated before loading on the gel. The requirement for concentration at this step was to precipitate proteins quantitatively in a solvent that would not interfere with electrophoresis. Gotti et al (1982) used a spacer of 14 cm length for loading the sample (the loading capacity was about 2.5 ml) and the concentration was carried out by the process of gel electrophoresis. The problem of this procedure was that the large volume loaded on the gel needed much longer time for sample focusing and the gel had to be prerun before loading the sample to allow the ion front to migrate a sufficient distance so that the large volume of sample could be focused properly. Even under these conditions, the polypeptide bands still showed a broad spreading appearance after electrophoresis which could decrease the recovery of the protein. Several other methods were tried and the problems were either the loss of protein sample during the concentration process or the concentrated sample containing factors such as high salt concentration which would interfere with electrophoresis. Finally, Wessel's precipitation method (1984) using methanol and

chloroform was found to be very effective for a fast, quantitative recovery of micrograms of proteins. The recovery was quantitative in a tested range of 2 ug to 20 ug proteins in our buffer system which included deoxycholate and methyl-α-D-mannopyranoside (Fig.21). The precipitated pellet could be dissolved in 1 x Laemmli loading buffer containing 5% SDS. This method allowed us to manipulate the volume of loading sample at will.

2. Electroblotting of polypeptide bands

Concentrated protein samples were separated on a 0.5 mm thick SDS polyacrylamide gel (10%) and electroblotted onto three layers of nitrocellulose filter in 80% Blotting Buffer:20% methanol (v/v). The standard blotting conditions of a constant current of 200 mA for two hours was tried first (Towbin et al , 1979). The blotted filters were stained with Ponceau S (Salinovich, et al, 1986) and no bands were seen on these filters. There were two possible explanations: one, that all proteins were still in the gel; the other, that proteins had passed through all layers of the membranes and went into the buffer. The gel was stained by silver and there was only a trace of residual proteins left. Thus , the protein bands must have entered the buffer under the blotting conditions. We then carried out a time course of the blotting from 5 minutes to 120 minutes. We found that the blotted proteins could be seen on the second layer of filters within 5 minutes and that the blotting pattern on the first layer of filter was uneven, i.e. the

transferring speed of smaller proteins was much faster than the larger proteins. The ideal situation would be that all proteins be evenly transferred and remained on first filter. Consequently, a low constant current of 20 mA for 16-20 hours was tried. We hoped that the low voltage would decrease the difference in the migration speed among different molecular bands and the retention force of the filter to the proteins would be sufficient to hold the proteins under such blotting conditions. Prestained protein markers covering a molecular weight range from 48 KD to 116 KD were tested for this low current blotting. The marker proteins were separated through a gel and the gel was scanned by a laser densitometer (Biomed Instruments). The integrated areas for each protein band were plotted as a control for later comparison. The gel was then electro-blotted at the low current condition described above onto two layers of filters and the first layer of the blotted filter was scanned. The integrated areas were plotted and compared with the integrated areas of the corresponding band scanned from the gel (Fig. 22). The results showed that the protein transfer was almost quantitative for those tested proteins with different molecular weights and that all these proteins remained on the first layer of the blotted filter. Since blotting behavior differs from protein to protein, we also tested whether this low current condition worked on our membrane preparation and the results were quite similar to the marker proteins (data not shown).

B. Tryptic Digestion

1. Tryptic digestion condition

To avoid the possible failure in sequencing by Edman degradation due to N-terminal blockage, which is not an uncommon case for proteins (Aebersold, 1987), and to obtain maximal information from the purified peptides, the strategy of internal amino acid sequencing was adopted. The major steps of the procedure are as follows: blotted protein bands are *in situ* digested by trypsin on the membrane; the tryptic fragments are separated by microbore reverse phase HPLC and fractions are collected; well separated fractions are sequenced by a gas phase sequencer to get internal amino acid sequences. The small amount of purified receptor made a high demand on maximizing the yield at each step.

Since the amount of trypsin used should be kept as low as possible to avoid high background generated by self-digestion of the enzyme, it was important to optimize the trypsin digestion conditions. Two types of buffer were compared in the presence and absence of 0.2% Ca++ and the trypsin activity was measured as described by Desnuelle (1966). The results (Fig.23) showed that the enzyme was stabilized by the presence of Ca^{++} . The buffer composed of 100 mM Tris.HCl, pH 8.2/ Acetonitrile, 95:5 (v/v) (Aebersold et al., 1987) is a better choice than 100 mM ammonium bicarbonate buffer pH 8.0 (Yuen et al., 1988). Under the condition of 1/20 (w/w) enzyme to protein ratio and 37°C overnight incubation, the digestion reached 80% completion when BSA was used as substrate (data not shown) .

2. Selection of Trypsin source

Impurities in trypsin will result in unexpected fragments and complicate the results. The purity of two batches of trypsin was compared by standard assay using bovine insulin B chain as substrate . The tryptic digests were separated by HPLC. The HPLC profile of tryptic fragments of insulin B by Sigma trypsin showed that the batch had some contamination which generated small fragments besides the two expected major peaks (Fig. 24). One batch of trypsin from Boeringer Mannheim was checked and used for its better quality (data not shown).

3. Tryptic digestion of the blotted polypeptide bands

Before the tryptic digestion, the blotted filters were coated with PVP-40 which was a necessary step to prevent the adsorption of trypsin to the filter that would cause a complete loss of enzyme activity in two hours (data not shown). The tryptic digestion was carried out in a siliconized miniglass tube to eliminate the adsorption of tryptic fragments onto the wall of the tube.

C. Reverse-Phase HPLC of tryptic cleavage fragments

The tryptic digests of protein bands were separated by reverse phase HPLC column (RP-300, 2.1 x 100 mm) using a gradient of 0-100% B as illustrated in Fig. 25 (A.B.C.). Panel A is the profile of tryptic digestion of the 57 kD band and panel B is the profile of tryptic digestion of the

44 kD band. A background of trypsin self-digestion in the presence of blank nitrocellulose membrane was shown in Panel C. These fractions were manually collected and major peaks were subjected to amino acid sequencing. However, it was not infrequently observed that one peak contained more than one peptide fragment. Therefore, other separation conditions were tested. Panel D shows a separation of 57 kD tryptic digest which differed from Panel A in buffer gradient (10 - 60 %B). Compared with panel A, peak number increased 0.5 fold. Panel E shows a separation of 44 kD tryptic digests which differed from Panel B in column diameter (1.0 x 100 mm) and buffer gradient of 10-60%. The fractions were monitored simultaneously both on the computer screen and the recorder. Compared with panel B, the number of peaks increased 2.4 folds which dramatically decreased the possibility of mixed fragments in one eluated peak. In the following experiemnts, we used the 1.0 x 100 mm column and buffer gradient 10-60% B for the separation of tryptic fragments.

D. Internal Amino Acid Sequencing

RP-HPLC separated peptide fractions were sequenced by either loading on a glass fiber filter directly or after lyophilizing without thawing the protein sample. The samples were transferred to an Applied Biosystems model 477A automated sequencer, operated according to manufacture's instruction. Stepwise liberated PTH-amino acids were identified using an on-line 120A PLC system equipped with a

PTH C18 column (Applied Biosystems)

Nine internal amino acid sequences obtained from several runs of HPLC separation are summarized in Table 6. These peptides were compared with published protein sequences deduced from putative cDNAs of nAChR from Drosophila (Hermans-Borgmeyer, 1986; Bossy, 1988; Sawruk, 1990). These cDNA clones were isolated based on the screening of Drosophila cDNA library with nAChR probes from other species. The protein structures and functions of these genes are not known yet. The comparison between the peptide sequences from our results and those deduced protein sequences showed in some cases considerable similarities between them with the highest similarity degree of 100% but none of them are 100% identical. These results provide primary evidence for the heterogeneity of the nAChR in Drosophila although a clear cut comparison can be made only after the cDNAs coding for these internal amino acid sequences are cloned and analysed .

Conclusion

An α-Bgt binding protein has been purified approximately 5,820 fold from Drosophila. The purified protein has strong affinity to nicotinic cholinergic ligands and the pharmacological profiles suggest that the protein is a functional nAChR. The method reported here allows for the purification of large amounts (in nanomole range) of receptor, sufficient for characterization of biochemical and physical properties, and internal amino acid sequencing.

The purified protein has a molecular weight of approximately 270,000 dalton, a sedimentation coefficient ($S_{20,w}$) of 9.4 S, a Stoke's radius of 7.4 nm and a f/fo of 1.72 which indicates that the protein is a transmembrane protein.

SDS PAGE analysis revealed that the 270 kD protein contains two types of subunits with molecular weights of about 44,000 and 57,000 daltons. Both peptides could be photoaffinity labeled in situ in the membrane and in the purified receptor sample providing evidence that these two protein bands are unlikely to be the result of proteolytic degradation during purification. The intact conformation of the receptor was maintained in the purified receptor sample.

Two types of binding sites were observed in the membrane preparation. The fact that the purified protein still contains two binding sites with K_d values of 0.20 nM and Kd 3.1 nM for α-Bgt suggests that the two binding sites were not separated after even more than 6000 fold purification after sucrose density gradient. However, the

change in the ratio of B_{max} between the two binding sites from 1:1 in membrane to 1:2 in solubilized preparation to 1:3 in the purified sample suggests the structural differences between the two binding sites. One alternative explanation is that the receptor contains two binding sites and one of which is easier to loss activity than the another. Whether the two binding sites could be separated needs to be further explored.

The purified proteins were separated by SDS PAGE and the two subunit peptide bands were electroblotted and digested with trypsin for internal amino acid sequencing. Nine independent sequences were obtained and nucleotide probes were designed for the cloning of the genes encoding the proteins. Several cDNAs were isolated from an adult Drosophila cDNA library (Appendix).

The muscle type nicotinic receptors are among the best characterized receptors (Claudio, 1989) which are composed of $\alpha_2\beta\gamma\delta$ with two α subunits binding to two ligands. However, the molecular structure of neuronal nicotinic receptors are still largely unknown. Breer et al.(1985, 1986) reported a single type of subunit composition of 65 kD for the locust ganglia nAChR with a molecular weight of 250 kD. It was suggested to be the ancestral AChR that is a homo-oligomer of α-like subunits that constitutes the prototype for the present vertebrate receptors. Neuronal nAChRs purified from vertebrates were mainly reported to be composed of two types of subunits. Components of 51 and 79 kD are obtained from rat brain nAChR by mAb affinity

purification (Whiting et al., 1986). The analysis of two subtypes of nAChRs purified from chick brain reveals components of 49 and 59 kD and 49 and 75 kD respectively (Whiting et al., 1987). Three types of subunits 49, 52 and 60 kD were reported for nAChR from chick ciliary ganglia in which two type combinations of subunits: 49 and 52 kD and 49 and 60 kD were suggested (Halvorsen, 1990). Our results do not favor the hypothesis by Breer. Rather, the Drosophila nAChR shows more similar structure to nAChRs in the vertebrate neuronal system. The distinct differences in the subunit composition of neuronal nAChRs with the muscle type nAChRs raises a considerable challenge with regard to the structure and functional relationship. What is the stoichiometry of the neuronal receptor ? It must be quite different from the muscle type nAChR. How many ligands could one receptor bind? The answers to these questions might shed light on a different mechanism of receptor function.

Three putative cDNAs coding for nAChR in Drosophila have been reported by using probes from muscle type nAChRs (Hermans-Borgmeyer et al 1986; Bossy et al, 1988; Sawruk et al., 1990). The function of these cDNAs is still not yet known. A thorough comparison between our clones and the cDNAs cloned are in progress. The combination of information on the receptor protein reported by this work and the molecular cloning results will provide a detailed picture of the structure of the neuronal nAChRs in Drosophila which might further suggest the diversity of neuronal nAChR in invertebrates.

FIGURE 1

Saturation curve of [^{125}I]-α-Bgt binding to membrane preparation from Drosophila heads. Duplicate assay mixtures containing increasing concentrations of [^{125}I]-α-Bgt and a constant amount of membrane sample (0.35 mg) were incubated for 1 hour at 23°C. Background binding at each toxin concentration was determined by preincubation with 10^{-4} M d-tubocurarine. Toxin binding was determined by the DEAE filter assay .

△ : total binding

▲ : background binding

o : specific binding calculated as total binding minus background binding.

Saturation Binding of Membrane Preparation

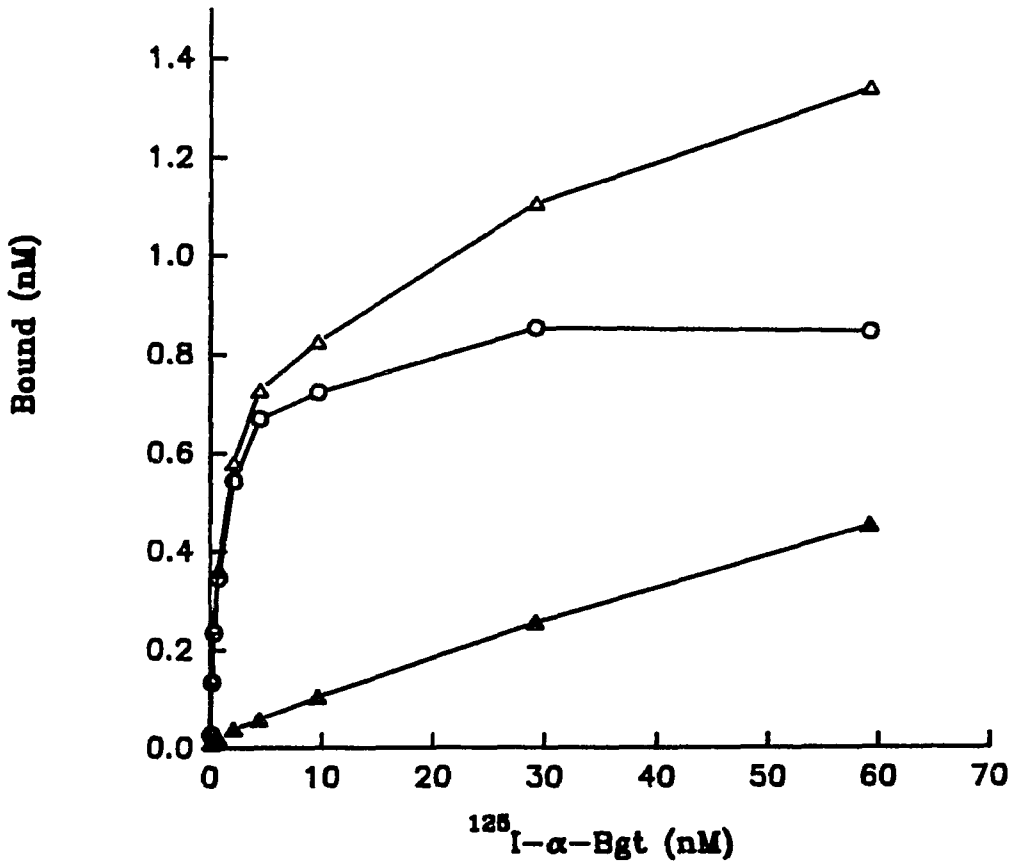


FIGURE 2

Scatchard plot of [^{125}I]-α-Bgt binding to Drosophila head membranes. The two site analysis of Scatchard plot for specific [^{125}I]-α-Bgt binding was carried out by using a Marquardt-Levenberg non-linear regression curve fitting procedure (Meeker et al., 1986) and performed by Sigmaplot (Version 4.0). The K_{d1} and K_{d2} are 4.1 x 10^{-10} M and 3.3 x 10^{-9} M respectively; B_{max1} to B_{max2} ratio is 1:1.

Scatchard Plot of Membrane Preparation

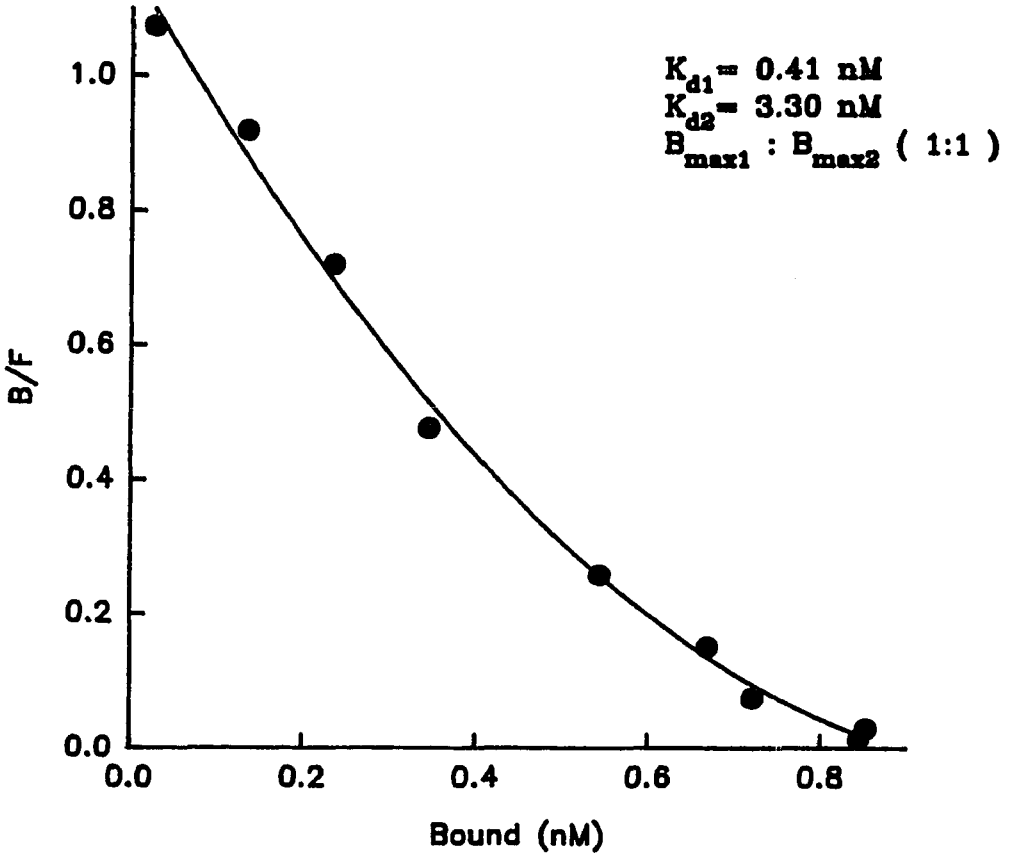


FIGURE 3

Inhibition of [^{125}I]-α-Bgt binding to membranes from Drosophila heads by various cholinergic ligands. The assays were carried out as described in Methods. The 100% value was defined as the [^{125}I]-α-Bgt binding in the absence of added ligand.

△: α-Bgt

▲: d-tubocurarine

○: atropine

▼: nicotine

■: acetylcholine

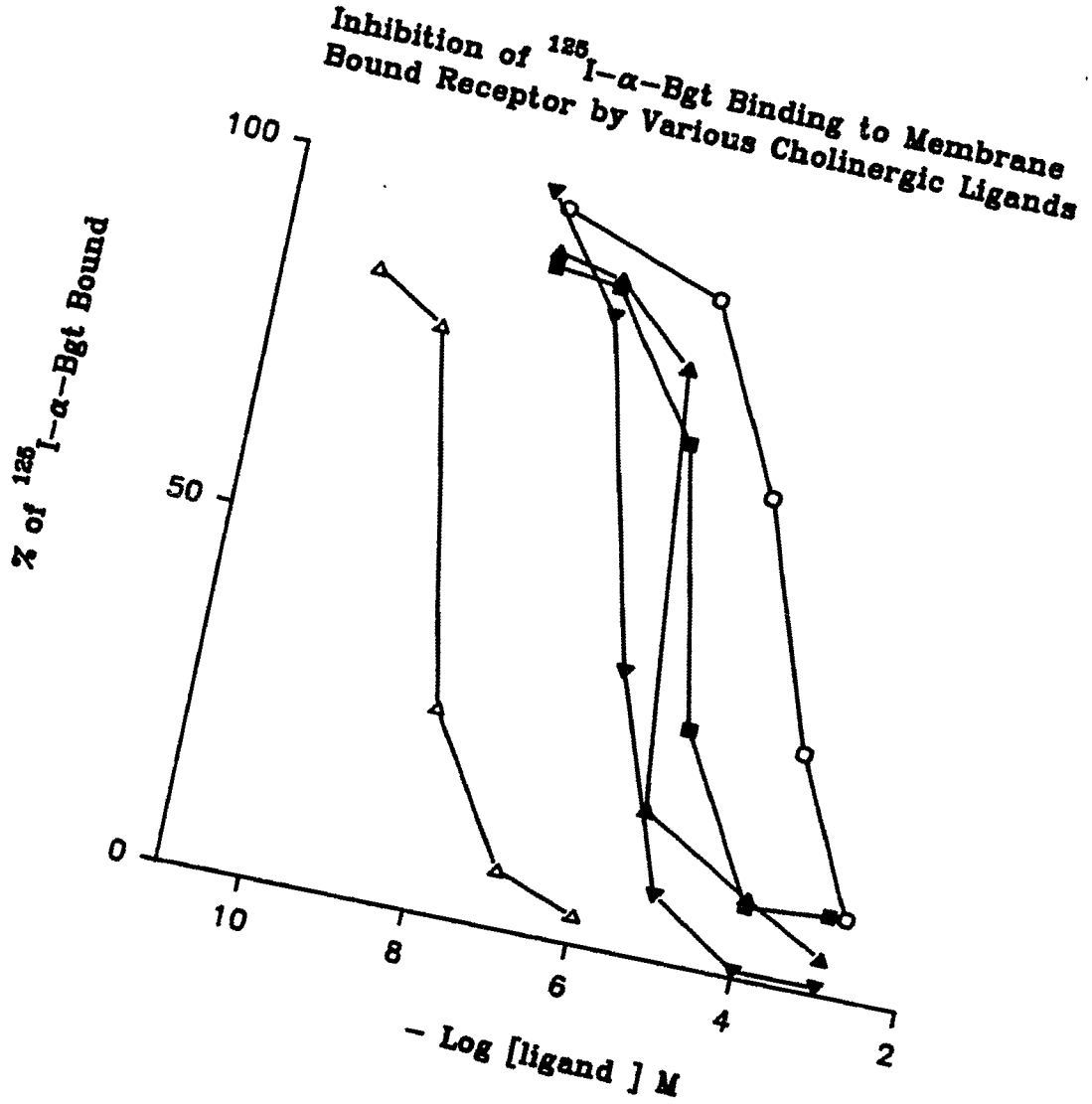

Inhibition of 125I–α–Bgt Binding to Membrane Bound Receptor by Various Cholinergic Ligands
% of 125I–α–Bgt Bound
100
50
0
10
8
6
4
2
– Log [ligand] M

FIGURE 4

Membranes from heads were photoaffinity labeled in situ and separated by SDS PAGE. An autoradiogram of a dried gel is shown. Two major labeled bands were detected after the autoradiography (lane 1: no drug). The molecular weight of these two bands are 57 kD and 44 kD after correction for the contribution of the bound [^{125}I]-α-bgt. The specificity of the photoaffinity label was tested by preincubation with 10^{-5} M of α-Bgt (lane 2: α-Bgtx).

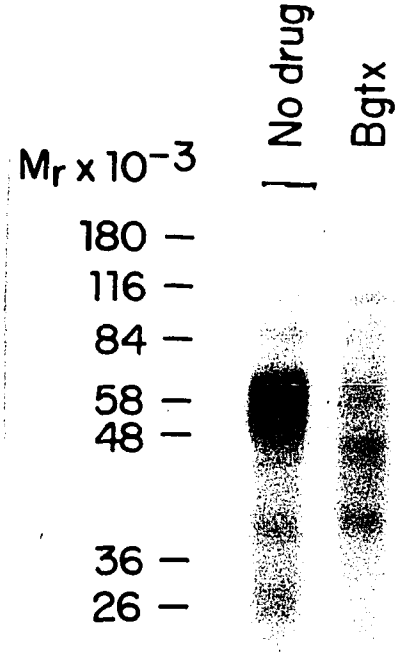

No drug
Bgtx
$M_r \times 10^{-3}$
180
116
84
58
48
36
26

FIGURE 5

The scheme for the purification of the nicotinic acetylcholine receptor from Drosophila is composed of three parts: the preparation of the sample for purification , the affinity purification and the concentration step.

Drosophila heads were used as starting material and homogenized in 100 mM salt buffer. The membranes were prepared by stepwise centrifugation and the receptor was solubilized in 0.2 M NaCl and 1.8% Triton X-100 - 0.6% deoxycholate at pH 8.5.

The affinity purification was carried out by loading the solubilized extract onto the α-Cobratoxin column. The column was washed with 100 mM NaCl and eluted with 0.2 M carbamylcholine. The eluate was further purified by loading onto a lentil lectin column. The column was washed with 1 M NaCl and eluted with 8% methyl-α-D-mannopyranoside .

The eluate from the lentil lectin column was concentrated in a Amicon concentrator by YM-100 membrane under 20 psi.

Protease inhibitors were included through out the whole purification process and all steps were carried out in 0-4°C. The purification process was monitored by specific activity.

SCHEME FOR THE PURIFICATION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR FROM Drosophila

Membrane preparation from Drosophila heads
(low salt buffer, proteases inhibitors)
⇓
Receptor solubilization
(0.2 M salt buffer, mixed detergents, PIM)
⇓
α-Cobratoxin affinity column
⇓
Lentil lectin affinity column
⇓
Amicon concentrator with YM-100 membrane
⇓
Purified receptor

FIGURE 6

Purification of α-Cobratoxin from Naja naja siamensis venom was as described by Cooper et al (1972). 1.5 g of venom was suspended in water and loaded onto a phosphocellulose column. The column was washed with 0.01 M K^+P_i buffer and eluted with a 0.01 M - 0.03 M K^+ buffer gradient. The eluate was precipitated with ammonium sulfate (68.4%, w/v) and the pellet was resuspended and loaded onto a Sephadex G-50 column. Fractions (15 ml) were collected and measured for protein concentration. The protein peak was pooled and loaded onto a the CM-52 column. The column was eluted with a NaCl gradient of 0.05 M-0.3 M Fractions of 15 ml were collected. Protein peak fractions were pooled, desalted onto a Sephadex G-50 column, lyophilized, resuspended and stored in -75°C.

The purified toxin was examed for its purity by reverse phase HPLC using a RP-300 column and the peak fraction was subjected to N-terminal amino acid sequencing.

PURIFICATION OF a-COBRA TOXIN FROM Naja naja siamensis VENOM

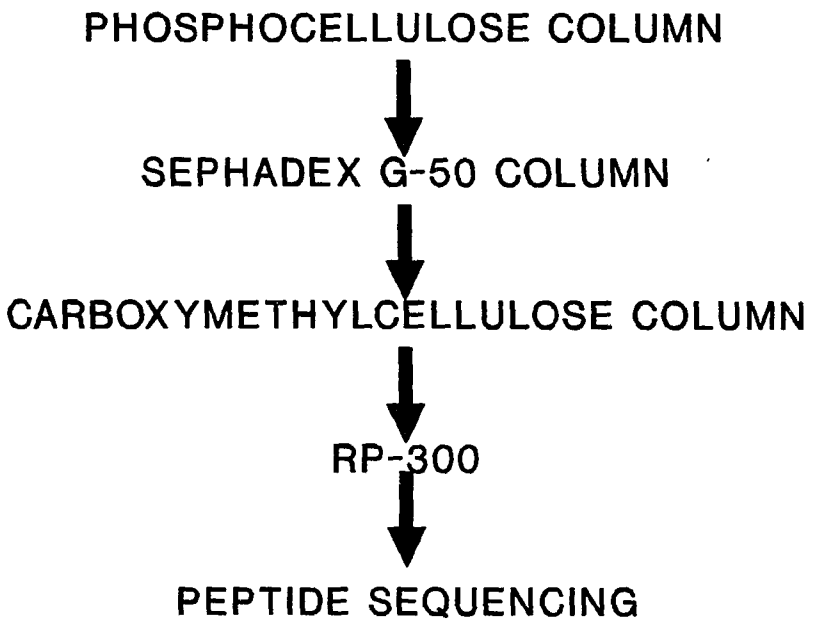


FIGURE 7

The purified α-Cobratoxin was checked by reversed phase HPLC column (RP-300) for its purity. A single peak appeared in the eluate from the column. The peak was subjected to 15 cycles of N-terminal amino acid sequencing. The data was compared with the α-Cobratoxin sequence reported by Tu (1973).

N-terminal Sequence Comparision of a-Cobrotoxin

IRCFITPDITSKDCP (Ann. Rev. Biochem. 42,235-258,1973)

IRCFITPDITSKDCP (Purified a-cobratoxin from the venom)

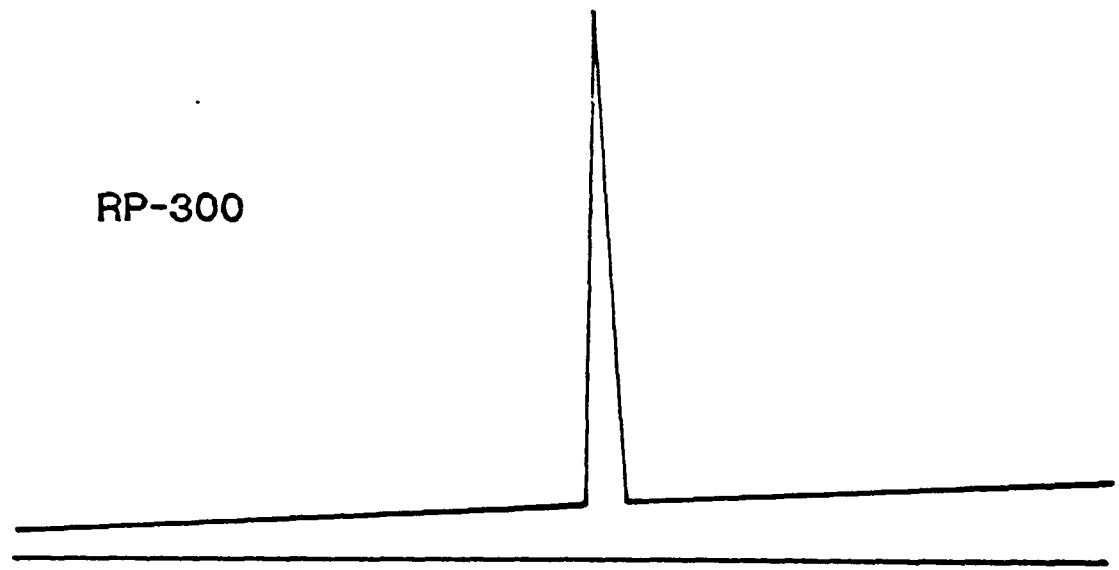


FIGURE 8

The inhibition curve of [^{125}I]-α-Bgt binding by the purified α-Cobratoxin to the membranes from heads. Assay conditions were as described in Materials and Methods.

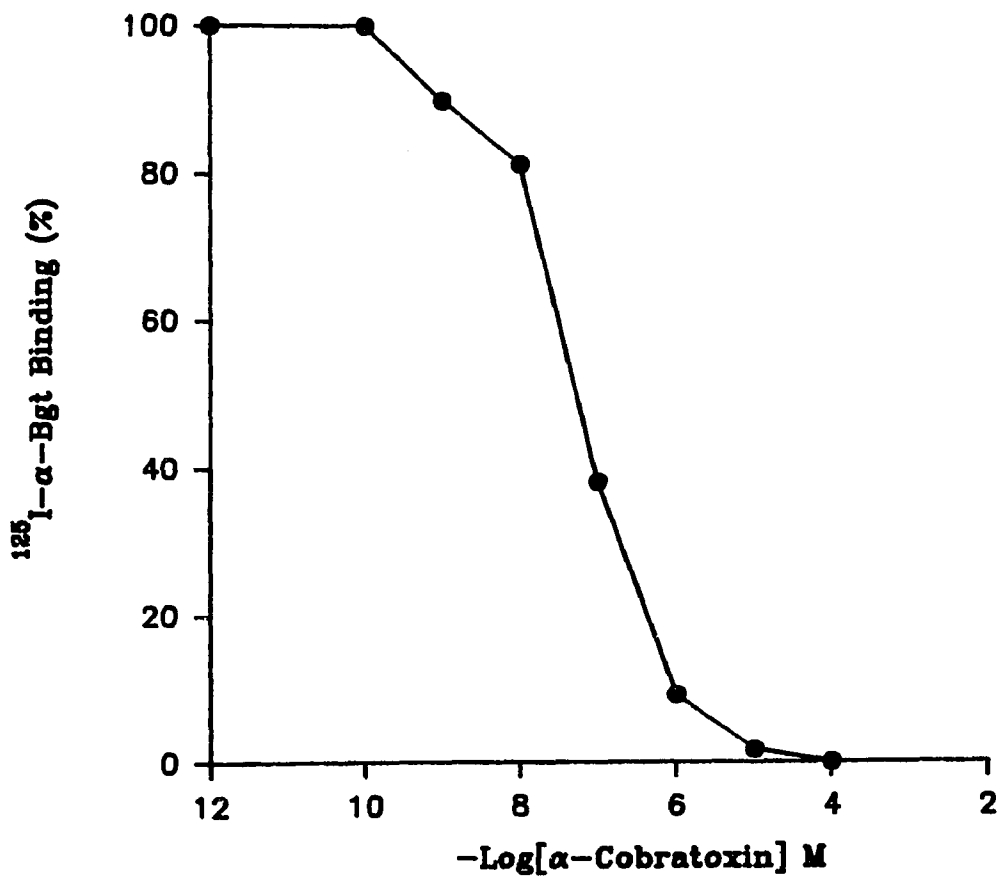

100
80
60
40
20
0
12
10
8
6
4
2
^{125}I-α-Bgt Binding (%)
-Log[α-Cobratoxin] M

FIGURE 9

The purification of lentil lectin from Lens culinaris was carried according to Howard (1971). The lentil lectin in the homogenate was first salted in and salted out by ammonium sulfate. A Sephadex G-75 column was used as an affinity column to purify lentil lectin. The column was washed with phosphate buffer (75 mM phosphate, 75 mM NaCl) and eluted with 2% methyl-α-D-mannopyranoside. The purified sample was analyzed by SDS PAGE (10%) and visualized by silver stain. A single protein band with an expected mw of 24.5 kD was detected.

PURIFICATION OF LENTIL LECTIN
From Lens culinaris

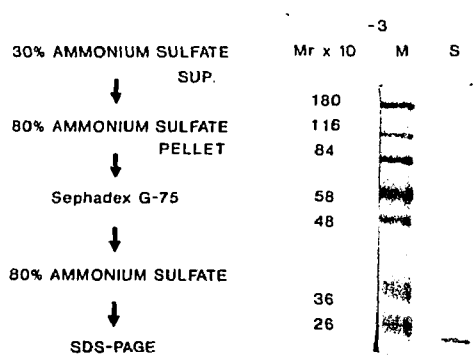


FIGURE 10

The effect of pH on recovery of α-Bgt binding activity was tested. Fly heads were homogenized in buffers with differing pH and assayed for the recovery of the binding activity. The results were compared to binding at pH 7.5 (as 100%).

Effect of pH on the recovery of α-Bgt binding sites from homogenate

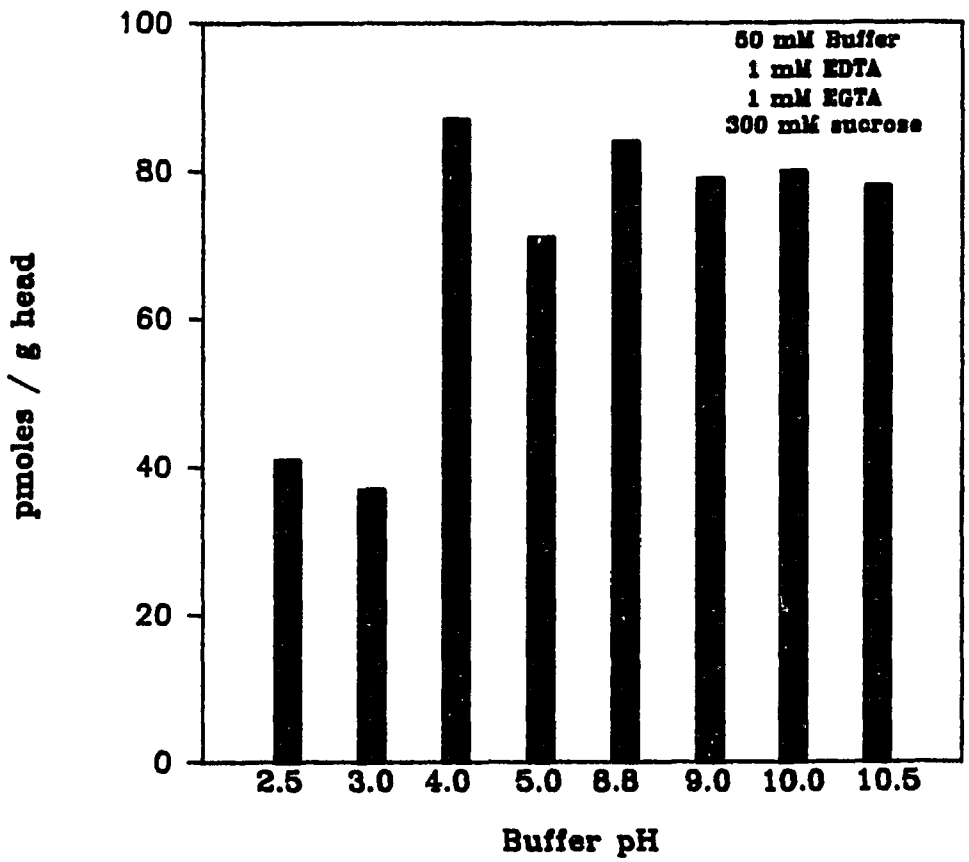


FIGURE 11

Scatchard plot of [^{125}I]-α-Bgt binding to the solubilized preparation. The two site analysis of Scatchard plot for specific [^{125}I]-α-Bgt binding was carried out by using a Marquardt-Levenberg non-linear regression curve fitting procedure (Meeker et al., 1986) performed by Sigmaplot (Version 4.0). The K_{d1} and K_{d2} values are 3.8×10^{-10} M and 2.9×10^{-9} M respectively; B_{max1} : B_{max2} ratio is 1:2.

Scatchard Plot of Solubilized Preparation

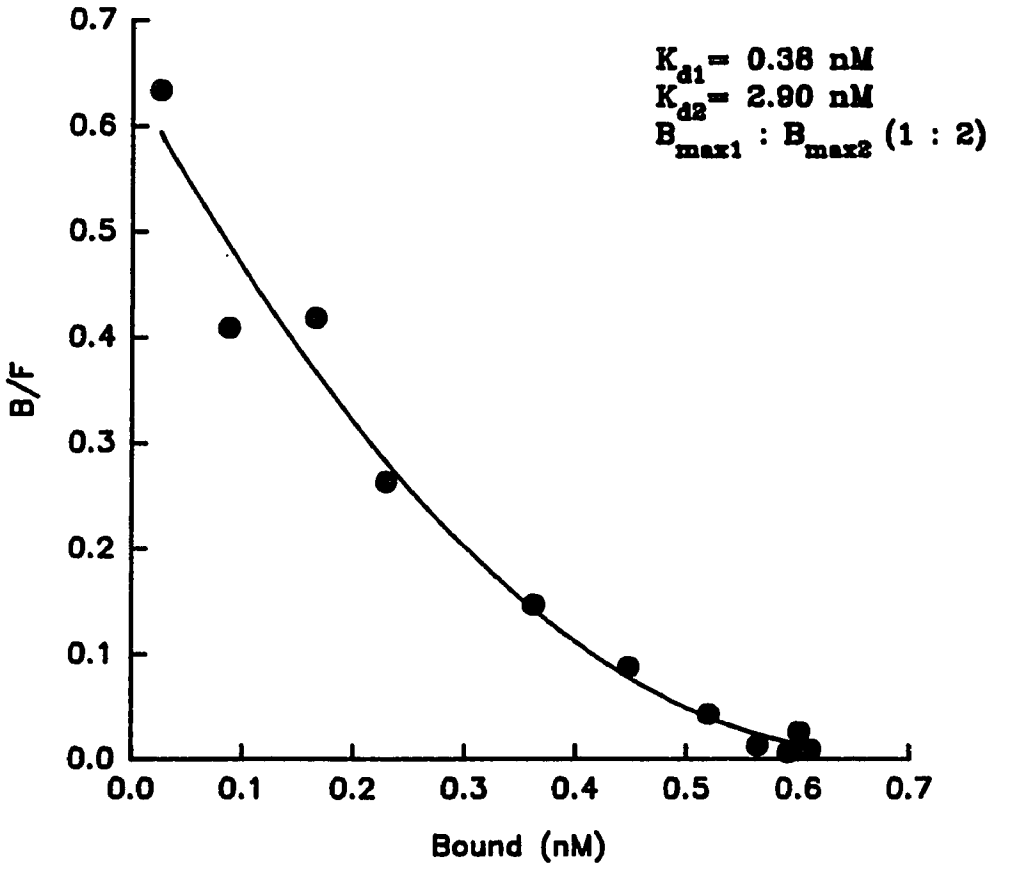


FIGURE 12

Purified receptor was either boiled in the presence of 1 x Laemmli loading buffer for 3 minutes or not boiled before loading onto the SDS gel. The stacking gel was included in the photo.

M : molecular marker.

S : purified receptor sample.

THE EFFECT OF HEATING
ON SAMPLE SEPARATION

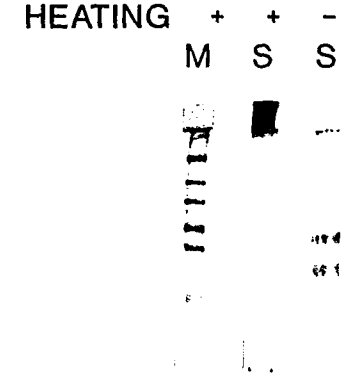


FIGURE 13

Receptors in the membrane fraction were photoaffinity labeled *in situ* as described in Methods. The labeled sample was spun for 2 minutes at full speed in a bench-top centrifuge after boiling for 3 minutes (left lane) or without boiling (right lane). The supernatant was loaded and separated by SDS PAGE. The gel was dried and exposed for autoradiography.

THE EFFECT OF HEATING ON SAMPLE SEPARATION OF in situ PHOTOLABELLED RECEPTOR

HEATING

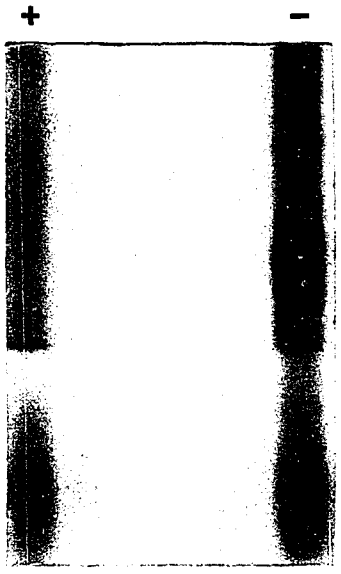


FIGURE 14

Silver stained 10% SDS polyacrylamide gel containing major fractions of the purification process. Same amount of protein (10 ug) was loaded for sample H. (homogenate); M.(membrane); S. (solubilized extract); 1E. (first column eluate). For 2E. (second column eluate, concentrated in a Amicon concentrator with a YM-100 membrane), 5 pmoles of binding sites were loaded.

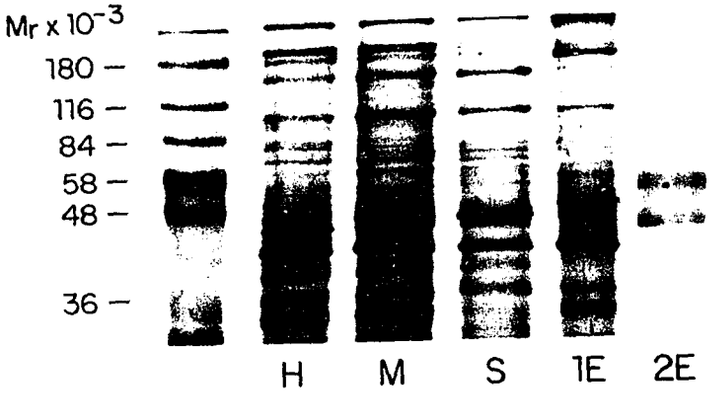

Mr x 10-3
180
116
84
58
48
36
H
M
S
1E
2E

FIGURE 15

Sucrose density gradient (5%-20%) analysis of purified receptor in the presence of protease inhibitors. 100 pmole of binding sites (200 ul) were loaded and centrifugated as described in Methods and Materials. Fraction sizes of 110 ul were collected and assayed for [^{125}I]-α-Bgt binding activity. Three groups of fractions were pooled as shown in the profile and proteins were precipitated and separated by a 10% SDS-PAGE. The silver stained gel is shown as the insert.

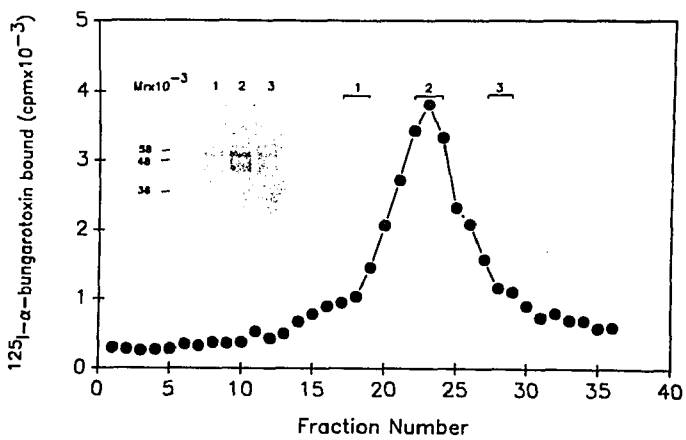

^{125}I-α-bungarotoxin bound (cpmx10^{-3})
Mrx10^{-3}
1 2 3
1
2
3
0
1
2
3
4
5
0 5 10 15 20 25 30 35 40
Fraction Number

FIGURE 16

Photoaffinity labeled receptors at various stages of purification were separated by 10% SDS-PAGE and exposed for autoradiography.

Lane 1: receptor labeled in situ in the membrane .

Lane 2: receptor labeled in situ in the membrane after preincubation with 10^{-5} M of cold α-Bgt.

Lane 3: receptor labeled in situ in the membrane, solubilized and purified by sucrose density gradient (5%-20%). Fractions were assayed for radioactivity and the peak fraction of the profile was used here for gel analysis.

Lane 4: labeling of purified receptor (concentrated lentil lectin eluate).

Lane 5: labeling of the pooled peak fractions from the sucrose density gradient centrifugation of the purified receptor (same sample as the pool 2 , lane 2 in Fig 15).

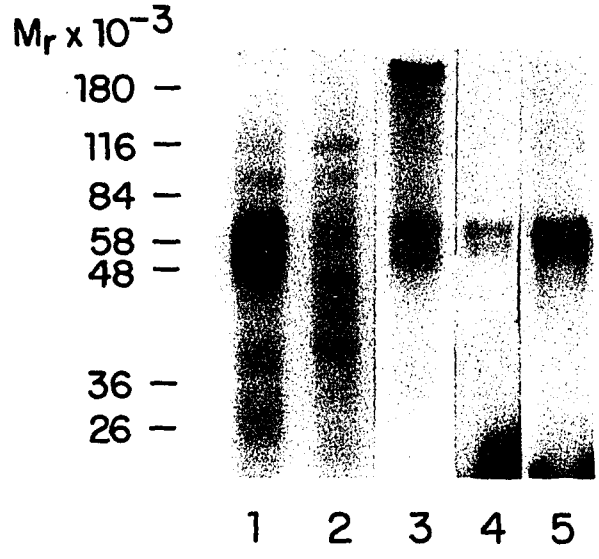

$M_r \times 10^{-3}$
180
116
84
58
48
36
26
1
2
3
4
5

FIGURE 17

Purified receptor was analyzed by gel filtration. 200 ul of the purified sample was loaded onto a Sepharose 4B column (1 x 50 cm) together with protein markers with known Stoke's radius. The flow rate was 248 ul/minute. Blue dextran was used for the determination of void volume. Fractions (575 ul) were collected and assayed for [^{125}I]-α-Bgt binding activity. The locations of the protein markers were determined by assays describes in Methods. The Stoke's radii of the marker proteins were plotted (Andrew, 1970) and the Stoke's radius of the receptor was determined from the plot.

Vo: void volume

thy: thyroglobulin

gal: β-galactosidase

fer: ferritin

cat: catalase

adh: alcohol dehydrogenase

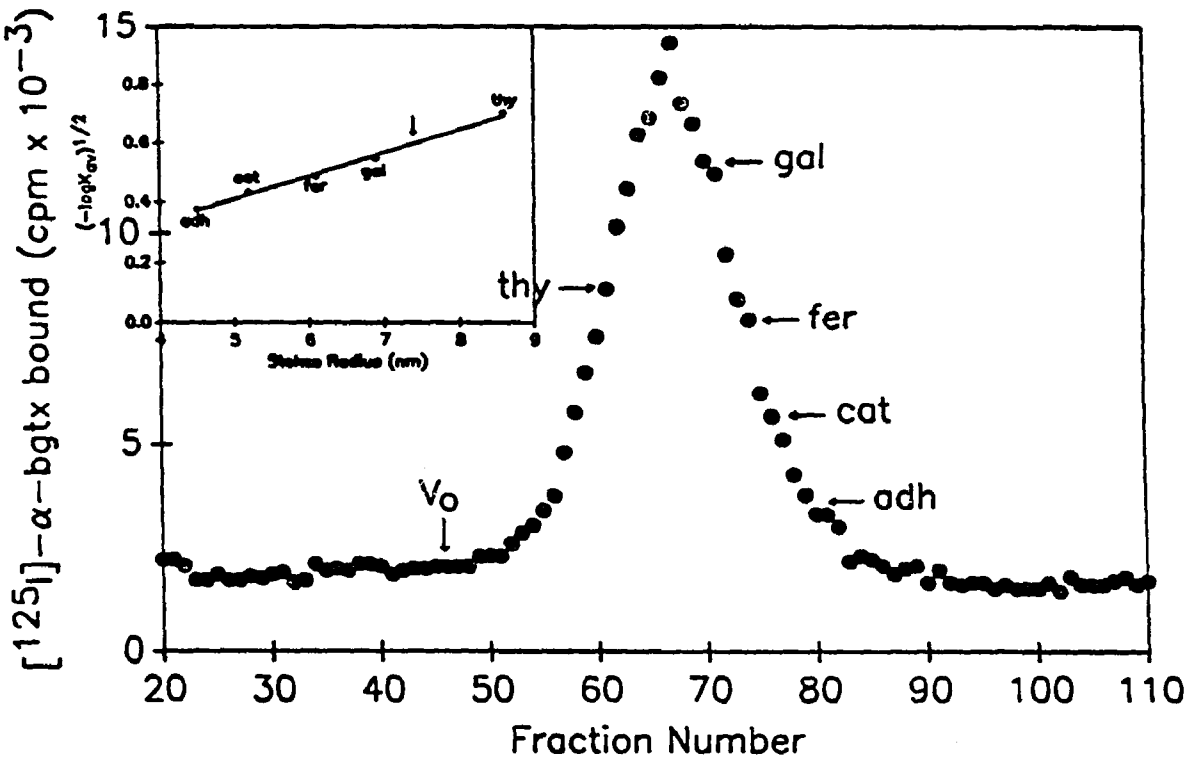

[125I]-α-bgtx bound (cpm x 10−3)
15
10
5
0
20
30
40
50
60
70
80
90
100
110
Fraction Number
thy
gal
fer
cat
adh
Vo

FIGURE 18

The sedimentation coefficient of purified receptor was determined by sucrose density gradient . The location of receptor in fractions was determined by [^{125}I]-α-Bgt binding activity and the locations of the protein markers of known sedimentation coefficient were determined by assays as described in Methods. The locations of the marker proteins were plotted against their sedimentation coefficient values and the $S_{20,w}$ value for purified receptor was determined from the plot (see arrow). For calibrating the contribution of bound detergent to the $S_{20,w}$ value of receptor, a parallel sucrose density gradient sedimentation was carried out in D_2O instead of standard H_2O together with the protein markers. The data were collected and plotted as above and the sedimentation coefficient of purified protein in D_2O was determined from the plot (see arrow).

gal: β-galactose

cat: catalase

adh: alcohol dehydrogenase

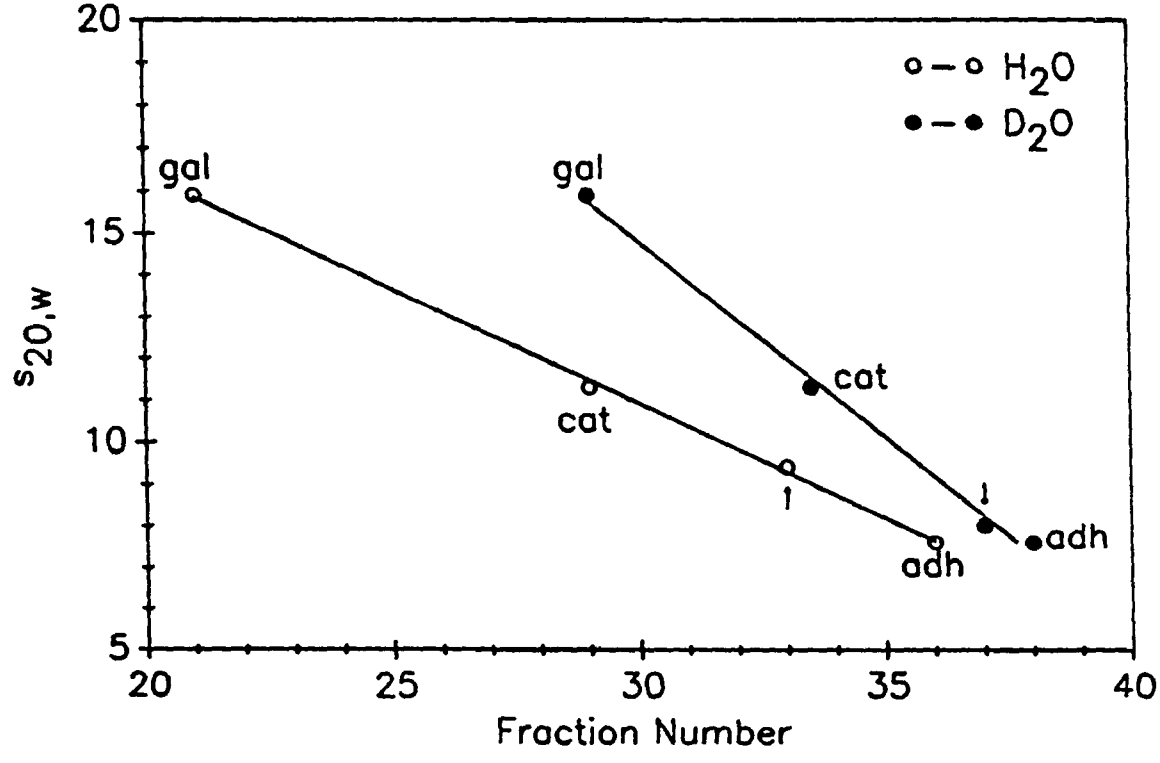

20
15
10
5
$s_{20,w}$
o—o H_2O
●—● D_2O
gal
gal
cat
cat
adh
adh
20
25
30
35
40
Fraction Number

FIGURE 19

Inhibition of [^{125}I]-α-Bgt binding to purified receptor by cholinergic ligands. The assays were carried out as described in Methods. The 100% was defined as the [^{125}I]-α-Bgt binding in the absence of added ligands.

▼: nicotine ▲: d-tubocurarine

■: acetylcholine o: atropine

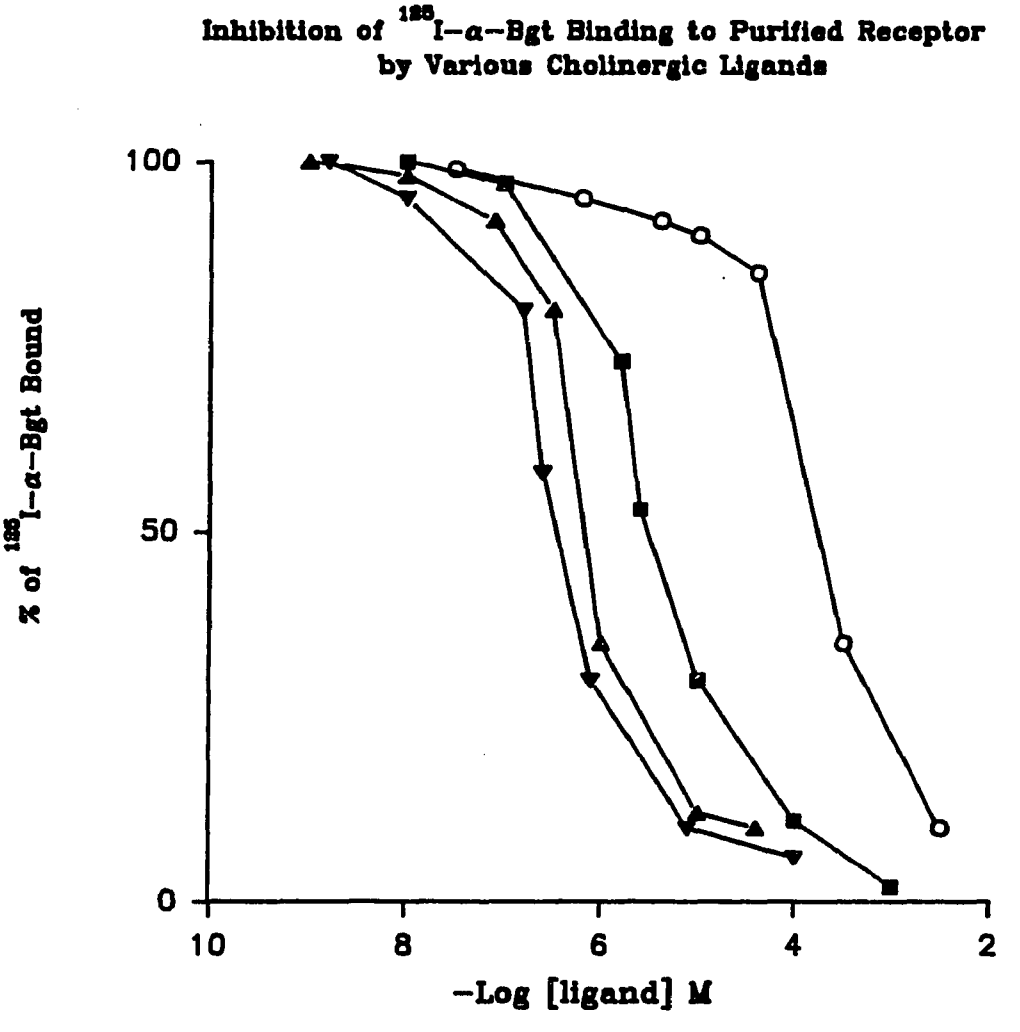

Inhibition of ^{125}I–α–Bgt Binding to Purified Receptor by Various Cholinergic Ligands
% of ^{125}I–α–Bgt Bound
100
50
0
10
8
6
4
2
–Log [ligand] M

FIGURE 20

Scatchard plot of [^{125}I]-α-Bgt binding to purified receptor. The two site analysis of Scatchard plot for specific [^{125}I]-α-Bgt binding was carried out by using a Marquardt-Levenberg non-linear regression curve fitting procedure (Meeker et al., 1986) and performed by Sigmaplot (Version 4.0). The K_{d1} and K_{d2} are 3.1 x 10^{-10} M and 2.03 x 10^{-9} M respectively . The B_{max1} to B_{max2} ratio is 1:3.

Scatchard Plot of Purified Receptor

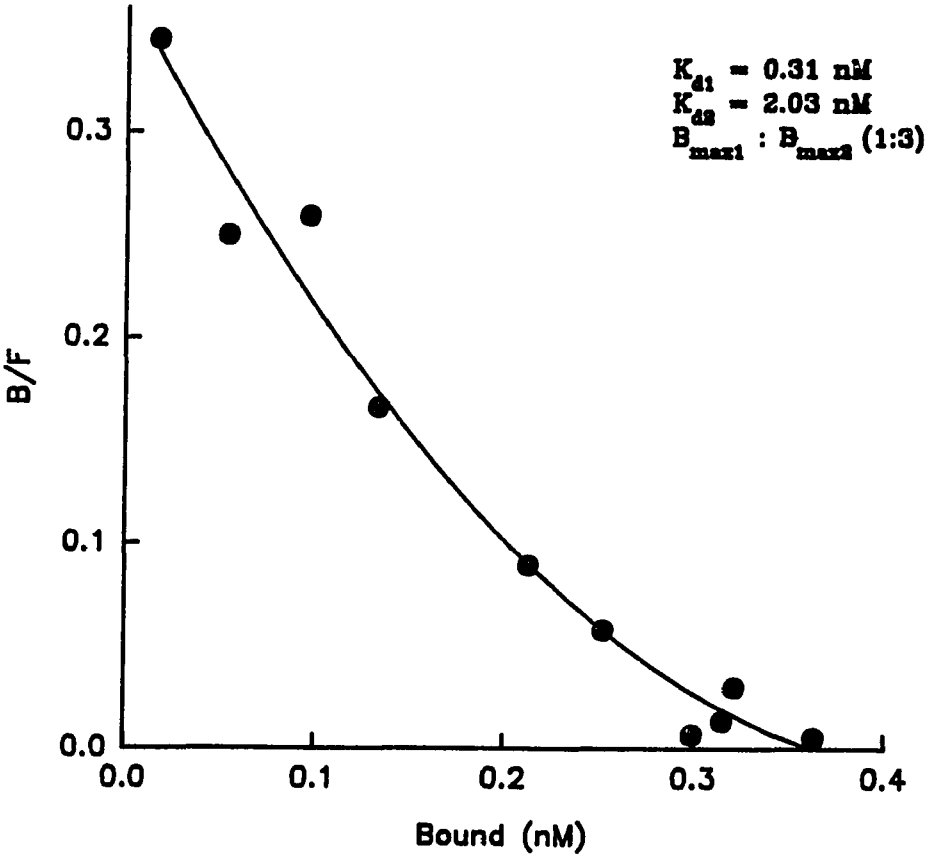


FIGURE 21

BSA was used to analyze the recovery of protein by the precipitation method (Wessel, 1988). Protein 2 ug, 5 ug, 10 ug and 20 ug were precipitated and resuspended in 1 x Laemmli loading buffer in 5% SDS before loading into the gel (■). In control, equal amounts of protein were directly loaded onto a SDS polyacrylamide gel (□). The gel was scanned by a laser densitometer (Biomed Instruments). The integrated areas for both types of samples were plotted for comparison.

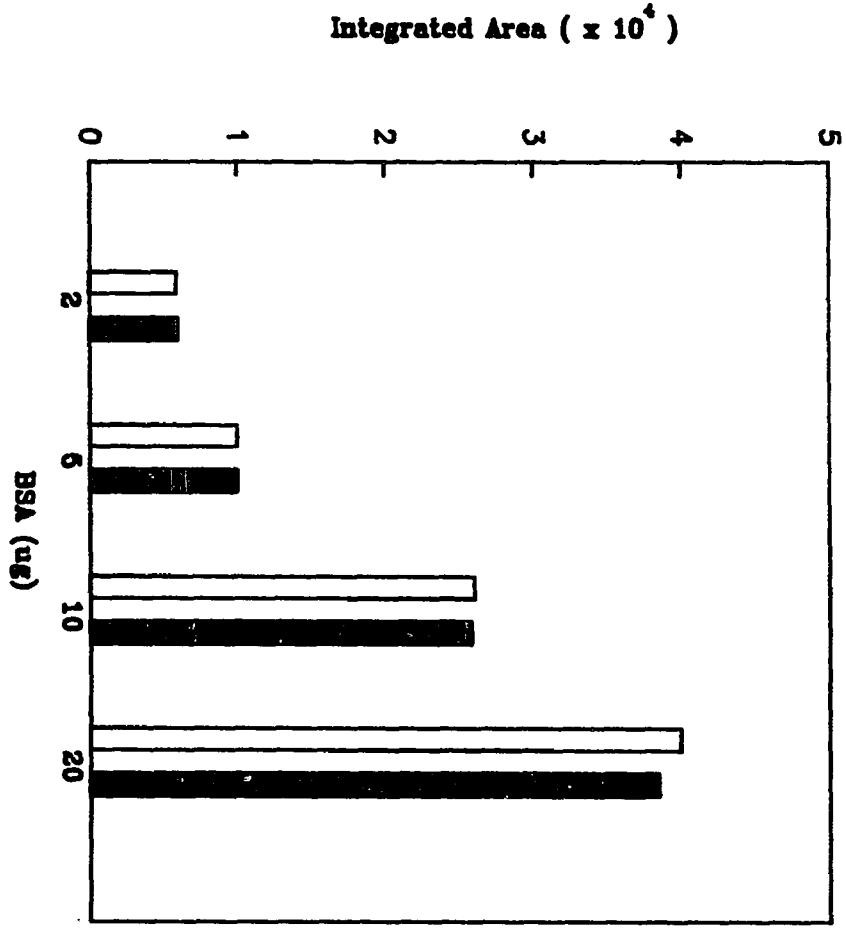

Integrated Area (x 10^4)
0
1
2
3
4
5
BSA (ug)
2
6
10
20

FIGURE 22

Prestained protein markers covering a molecular range from 48 kD to 116 kD were tested under the low voltage blotting condition (25 volts). The marker proteins were first separated by SDS PAGE and the gel was scanned by a laser densitometer (Biomed Instruments). The integrated areas for each protein band was plotted (□) as a control for later comparison. The gel was then electro-blotted onto two layers of filters and the first layer of the blotted filter was scanned. The integrated areas were plotted (■) and compared with the integrated areas of the corresponding band scanned from the gel.

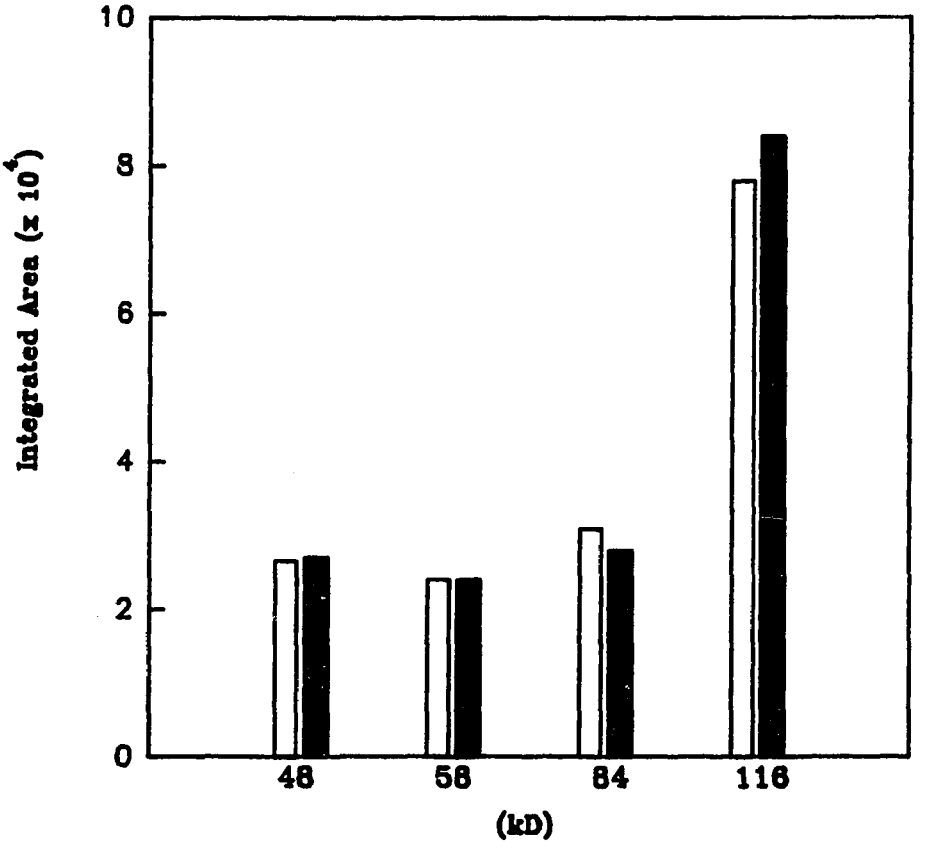

10
8
6
4
2
0
Integrated Area (x 10⁴)
48
58
84
116
(kD)

FIGURE 23

The trypsin digestion conditions were evaluated by measuring the enzyme activity (Desnuelle, 1966) in the presence and absence of 0.2% Ca^{++}.

▲: 100 mM Tris.HCl, pH 8.2 /acetonitrile 95:5 (v/v), 0.2% Ca^{++}.

△: Buffer as above without Ca^{++}

•: 100 mM ammonium bicarbonate pH 8.0 with 0.2% Ca^{++}

o: Buffer as above without Ca^{++}.

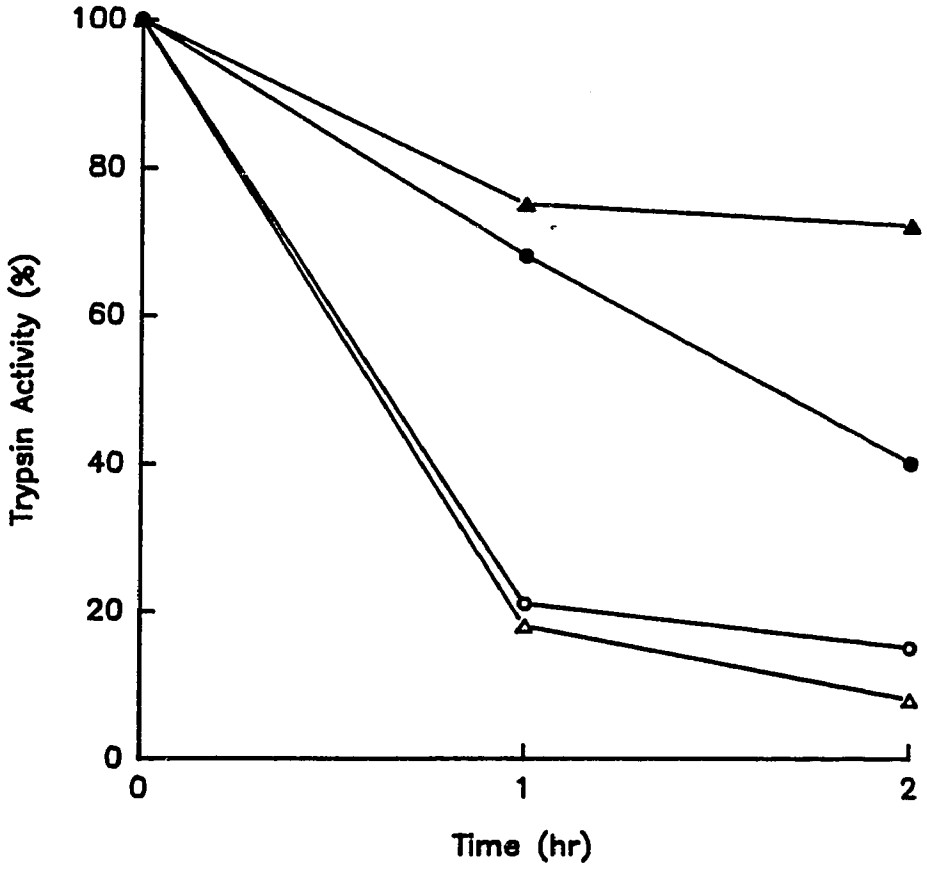

100
80
60
40
20
0
Trypsin Activity (%)
0
1
2
Time (hr)

FIGURE 24

Insulin B was digested by trypsin from Sigma to test the purity of that batch of trypsin. The tryptic fragments were separated by HPLC and the profile was compared with the reported tryptic map of bovine insulin B (Sigma). The two major peaks are the expected peaks while other smaller peaked might result from contaminations in that batch of trypsin.

Selection of Trypsin Source

HPLC profile of tryptic fragment of insulin B chain

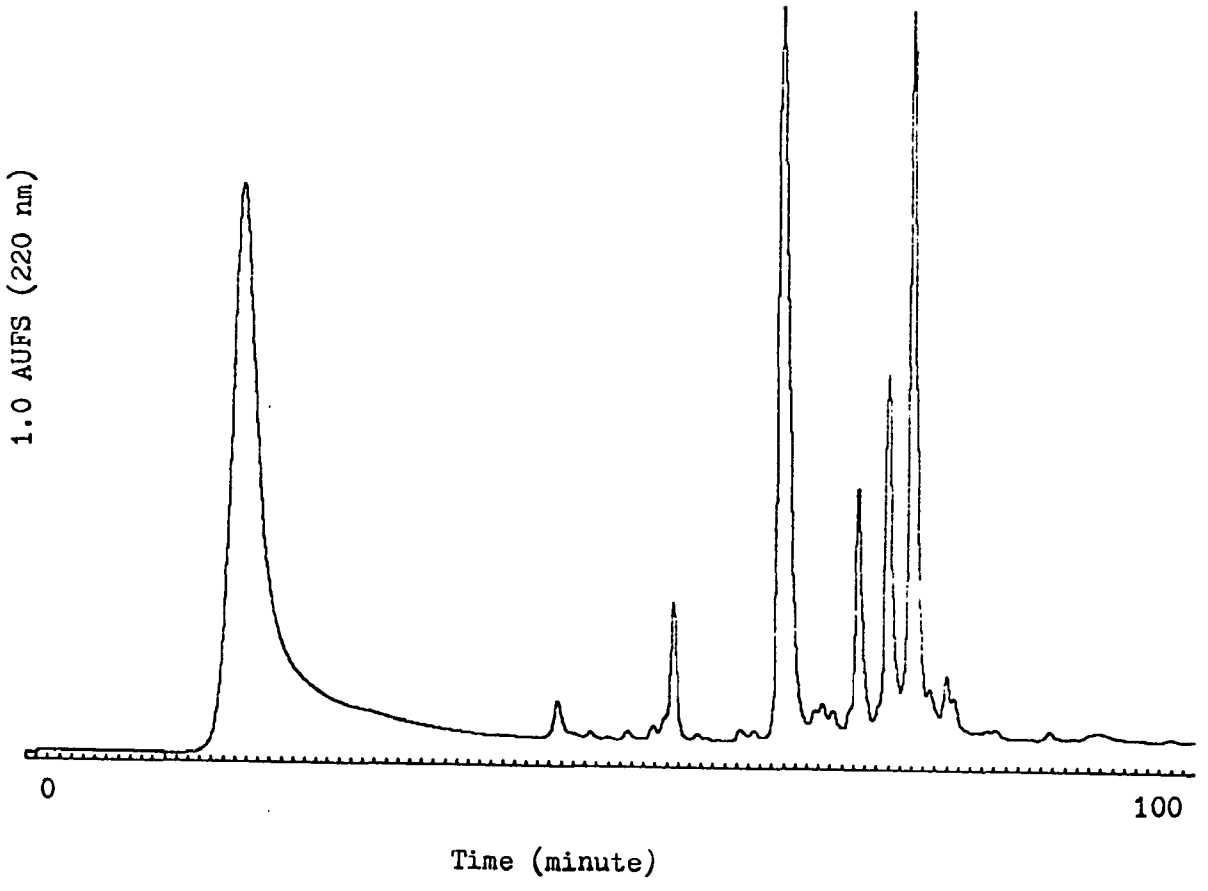


FIGURE 25

The separation conditions of tryptic cleavage fragments by reverse-phase HPLC were compared. Buffer A is 0.1% TFA. Buffer B is 0.08-0.095% TFA in acetonitrile/H_2O, 70:30 (v/v).

A. column RP-300, 2.1 x 100 mm, 0-100% B, 57 KD band.
B. column RP-300, 2.1 x 100 mm, 0-100% B, 42 KD band.
C. column RP-300, 2.1 x 100 mm, 0-100% B, trypsin self-digestion in the presence of blank nitrocellulose filter.
D. column RP-300, 2.1 x 100 mm, 10-60% B, 57 KD band.
E. column RP-300, 1.0 x 100 mm, 10-60% B, 42 KD band.

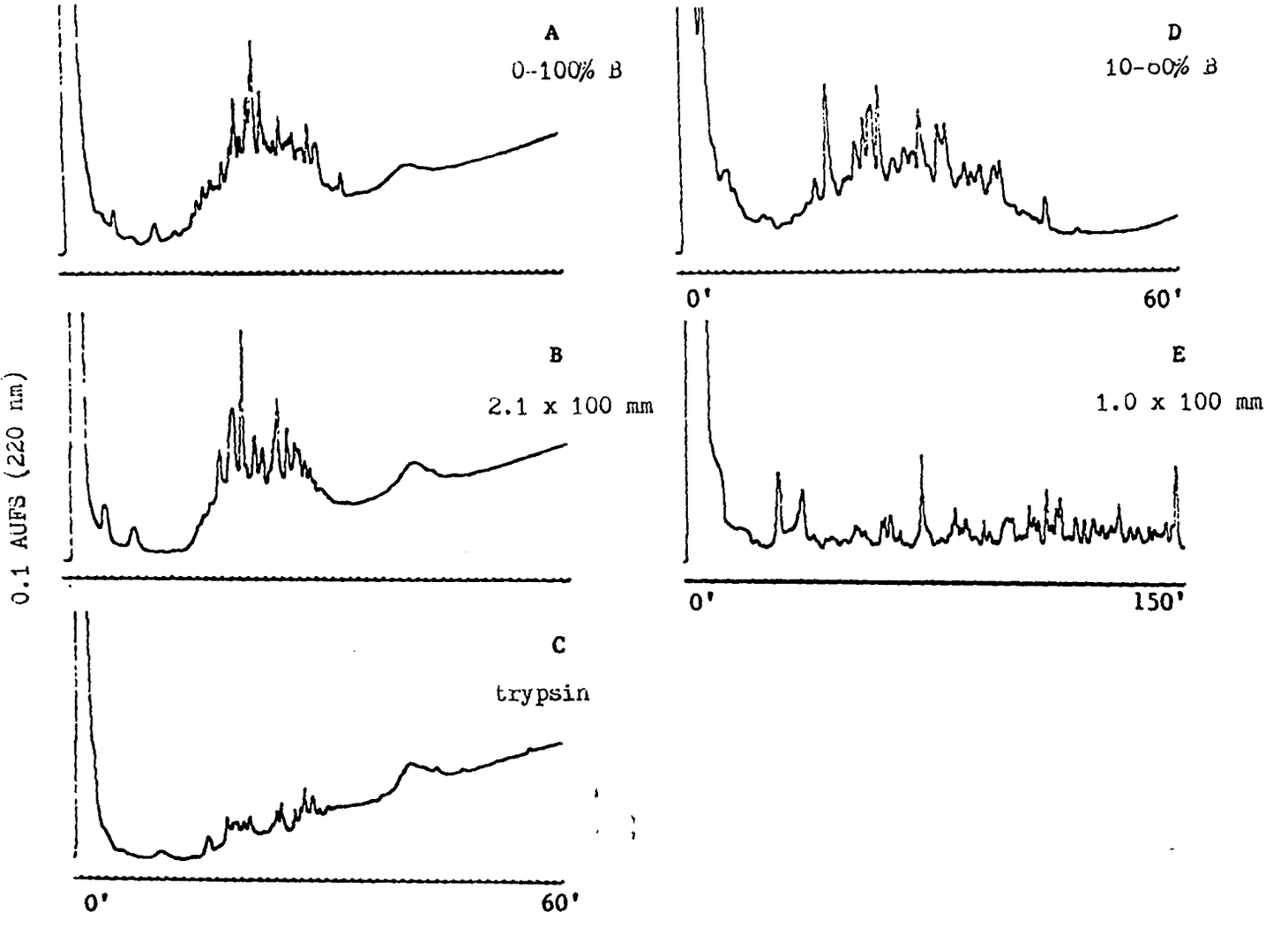

A
0-100% B
D
10-00% B
0'
60'
B
2.1 x 100 mm
E
1.0 x 100 mm
0.1 AUFS (220 nm)
0'
150'
C
trypsin
0'
60'

TABLE 2

Summary of [^{125}I]-α-Bgt binding to Drosophila membranes, solubilized extract and purified receptors. The data were calculated from equilibrium binding experiments as shown in Fig.2, Fig. 11 and Fig. 20.

Summary of [^{125}I]-α-Bgt Binding

Preparation	K_{d1} (nM)	K_{d2} (nM)	B_{max1} / B_{max2}
Head membranes	0.41	3.30	1 : 1
Solubilized extract	0.38	2.90	1 : 2
Purified receptor	0.31	2.03	1 : 3

TABLE 3

Comparison of the pharmacological specificities of receptor before and after purification. IC_{50} is defined as the concentration of ligand required to inhibit [^{125}I]-α-Bgt binding by 50%. The profiles of the inhibition experiments were shown in Fig. 3 and Fig. 19.

IC_{50} of Various Cholinergic Ligands for Binding to the Receptor

Ligand	Membrane-bound Receptor (M)	Purified Receptor (M)
Nicotine	4.5×10^{-7}	5.0×10^{-7}
d-Tubocurarine	2.6×10^{-6}	8.4×10^{-7}
Acetylcholine	2.8×10^{-6}	3.6×10^{-6}
Atropine	5.7×10^{-5}	1.6×10^{-3}

TABLE 4

The summary of a purification of a receptor from 28 g of Drosophila heads. Protein amount(*) was determined by amino acid analysis.

Purification of the Neuronal Nicotinic Acetylcholine Receptor

Fraction	[^{125}I]-α-Bgt binding (pmol)	Recovery (%)	Protein (mg)	Spec. Activit (umol/g protein)	Purifcation -fold
Homogenate	2281	100	3326	0.00067	-
Triton X-100 solubilized	453	19	273	0.00166	2.4
Cobratoxin I	210	9.2	1.3	0.156	232
Lentil lectin	106	4.6	0.056*	1.90	2835
Amicon YM100 Concentrate	45	2.0	0.0113*	3.90	5820

This purification is based on a starting amount of 28g of Drosophila heads.

* Protein determination carried out by amino acids analysis

TABLE 5

Summary of the biophysical properties of the purified receptor.

Biophysical Properties of Purified nAChR

$S_{20,w}$	9.4 S
Stokes radius	7.4 nm
f/fo	1.72
Partial specific volume	0.768 ml/g
M_r of receptor-Triton X-100 complex	333,000 dalton
M_r of receptor	270,000 dalton
Subunit composition	57,000 dalton 44,000 dalton

TABLE 6

The summary of internal amino acid sequences from two protein bands and the complimentary oligonucleotide probes synthesized.

FRACTION	PEPTIDE SEQUENCE	OLIGONUCLEOTIDE PROBE
44 kD		
B9	WIETEY	TACTCGGTCTCGATCCA A A
G27	IQNYGGVR	CCGTAGTTCTGGAT A
M27	ADEGFDGTYQIN	GTTGGTCTGGTAGGT A A
M55N49	LWKPDVLMY	1 GTACATCAGCACGTCGGGCTTCCACAG G G A T 2 GTACATCAGCACGTCGGGCTTCCACAG G A A T
N53	YHDIVKNRIIRMQK	CTTCTGCATGCGGATGATGCGGTTCTT A A A A
C15	IQSVELVSDIEPIESSR	**
57 kD		
D17	GEFDVNPLD	GGGTTCACGTCGAACTC G A
D18	FTTDQLPDVL	GGCAGCTGGTCGGTGATGAA G A A A
D27	VLDIDLDFR	1 CGGAAGTCCAGGTCGATGTCCAG A A A A G 2 CGGAAGTCGAGGTCGATGTCCAG A A A A G

** No probe was synthesized due to the high degree of degeneracy.

APPENDIX

Oligonucleotide Designing and Screening cDNA Library

A. Oligonucleotide Probe Design

The degeneracy of the probes designed was decreased by several strategies. First, two published putative cDNA clones, ARD and ALS, for neuronal nicotinic acetylcholine receptor from Drosophila (Hermans-Borgmeyer, 1986; Bossy, 1988) were analyzed for the codon frequencies. The result was compared with codon frequency tables for Drosophila genes (Sharp, 1988) in which codon usage frequencies were classified into two categories: highly expressed genes and low expressed genes. We found that the codon usage patterns of the two cDNAs matched pretty well with the highly expressed gene pattern. Thus, the highly expressed codon table was used as a parameter in the backtranslation of the amino acid sequences by backtranslation program designed by the Genetics Computer Group at University of Wisconsin (Devereux., 1984). To further decrease the degree of degeneracy, we analyzed the backtranslated nucleotide sequences by comparing then with the codon usage patterns of ARD and ALS to eliminate degenerated codons with frequencies less than 15%.

All probes were summarized in Table 6. These are the complimentary sequences of those derived from the peptide sequences. These oligonucleotide probes were synthesized and used for screening.

B. Screening of the cDNA Library

A Drosophila cDNA library was screened with ^{32}P-labeled oligonucleotide probes. After repeated screening with increasing stringency , several positive clones were isolated. Some positive clones were subcloned into a pBluescript vector for further analysis. One representative southern blot is shown in Fig. A-1 in which two fragments of molecular weight about 2.3 Kb and 3.0 Kb hybridized strongly with the probe 41 corresponding to the fraction M27 sequence while two control DNAs showed negative signal. The sequencing and further characterization of these cDNAs are in progress.

FIGURE A-1

The probe 41, GTTG(A)GTCTGGTAG(A)GT (from sequence M27), was used in this Southern blotting under conditions as described in Methods.

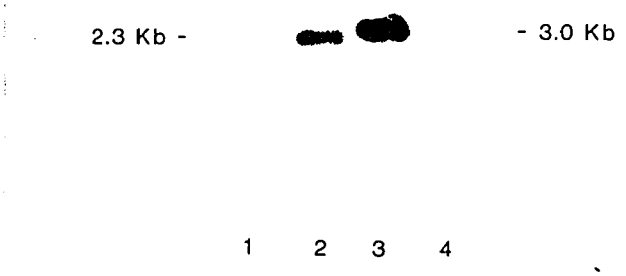
Lane 1: control HindIII cut λ DNA

Lane 2: clone 41-1 EcoR I fragment

Lane 3: clone 41-2 EcoR I fragment

Lane 4: control plasmid pIBI 31

Southern Blotting



Probe 41 GTTG(A)GTCTGGTAG(A)GT

1. Hind III cut lamda DNA
2. clone 41-1 EcoR I fragment
3. clone 41-2 EcoR I fragment
4. plasmid pIBI 31

REFERENCES

Aebersold, R.H., Leavitt, J., Saavedra, R.A. and Hood, L.E. (1987) Internal amino acid sequence analysis of proteins separated by one or two dimensional gel electrophoresis after in situ protease digestion on nitrocellulose. Proc. Natl. Acad. Sci. USA 84, 6970-6974.

Andrews, P. (1970) Estimation of molecular size and molecular weight of biological compounds by gel filtration. In Methods of Biochem. Analysis. Ed. Glick, D., J. Wiley & Sons, N.Y. Vol. 18, pp 1-53 .

Ben-Barak, J. B. and Dudai, Y. (1979) Cholinergic binding sites in rat hippocampal formation : properties and ontogenesis. Brain Res. 166, 245-257.

Blanchard, S.G., Quast, U., Reed, K., Lee, T., Schimerlik, M.I., Vandlen, R., Claudio, T., Strader, C.D., Moore, H-P.H., and Raftery, M.A. (1979) Interaction of [^{125}I]-α-bungarotoxin with acetylcholine receptor from Torpedo californica. Biochemistry 18, 1875-1883.

Bossy, B., Ballivet, M., and Spierer, P. (1988) Conservation of neural nicotinic acetylcholine receptors from Drosophila to vertebrate central nervous systems. EMBO J. 7, 611-618

Breer, H. (1981) Comparative studies on cholinergic activities in the central nervous system of Locusta migratoria. J. Comp.Physiol. 141, 271-275.

Breer, H., Kleene, R. and Benke, D. (1985) Molecular forms and subunit structure of the acetylcholine receptor in the central nervous system of insects. J. Neurosci. 5, 3386-3392.

Breer, H., Hinz,G., Madler,U. and Hanke, W.(1986) Identification and reconstitution of a neuronal acetylcholine receptor from insects. In NATO ASI Series H: Cell Biology, Volume 3, Nicotinic Acetylcholine Receptor. A. Maelicke (ed.), Springer-verlag, Berlin, pp. 319-332.

Breer, H., and Sattelle, D.B., (1987) Molecular properties and functions of insect acetylcholine receptors. J. Insect. Physiol. 33,771-790.

Callec, J.J. (1985) Synaptic transmission in the central nervous system. In " Comprehensive Insect Physiology, Biochemistry and Pharmacology" (eds. Kerkut, G.A. and Gilbert, L. I.) Vol. 5, pp. 139-179, Pergamon Press, New York.

Changeux, J.P., Giraudat, J., and Dennis, M. (1987) The nicotinic acetylcholine receptors: molecular architecture of a ligand-regulated ion channel. Trends Pharmacol. Sci. 8, 459-465.

Clarke, S. (1975) The size and detergent binding of membrane proteins . J. Biol. Chem. 250, 5459-5469.

Clark, P.B.S. (1987) Recent progress in identifying nicotinic cholinergic receptors in mammalian brain. Trends Pharmacol.Sci., 8, 32-35.

Claudio T. (1989) Molecular genetics of acetylcholine receptor-channels. In Frontiers in Molecular Biology: Molecular Neurobiology, D.M.Glover and B.D.Hames, eds. (Oxford: IRL Press), pp. 63-142.

Clementi, F., Gotti, C. and Sher, E. (1986) Nicotinic Acetylcholine receptors in the Nervous System, NATO series, series H: Cell Biology, 25.

Colquhoun, D., Ogden, D.C. and Mathie, A. (1987) Nicotinic acetylcholine receptors of nerve and muscle: functional aspects. Trends Pharmacol. Sci. 8, 465-472.

Conti-Tronconi, B.M., Dunn, S.M.J., Bernard, E.A., Dolly, J.O., Lai,F.A., Ray, N. and Raftery, M.A. (1985) Brain and muscle nicotinic acetylcholine receptors are different but homologous proteins. Proc. Natl. Acad. Sci. USA.82, 5208-5212.

Cooper, D. and Reich, E. (1972) Neurotoxin from venom of the Cobra, Naja naja siamensis-purification and radioactive labeling. J. Biol. Chem. 247, 3008-3013

Devereux, J., Haeberi, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX.Nucleic Acids Reseach 12(1), 387-395.

Dudai, Y. (1978) Properties of an α-bungarotoxin-binding cholinergic nicotinic receptor from Drosophila melanogaster. Biochim. Biophys. Acta 539, 505-517.

Dudai, Y., and Amsterdam, A. (1977) Nicotinic receptor in the brain of Drosophila melanogaster demonstrated by autoradiography with [^{125}I]-α-bungarotoxin. Brain Res. 130, 551-555.

Gepner, J.I. (1979) Purification and molecular and pharmacological properties of an α-bungarotoxin binding component from insects. Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA.

Giacobini, E., DeSarno, P., MaIlhany, M. and Clark, B. (1988) The cholinergic receptor system in the frontal lobe of Alzeimer patients. In Nicotinic acetylcholine receptors in the nerveous system (eds. Clementi, F., Gotti, C. and Sher, E.) NATO ASI Series H: cell Biology, Vol. H 25, pp. 367-378.

Gotti, C., Conti-Tronconi, B. M. and Raftery, M.A.(1982) Mammalian muscle acetylcholine receptor purification and characterization. Biochem. 21, 3148-3154.

Halvorsen, S. W. and Berg, D.K. (1990) Subunit composition of nicotinic acetylcholine receptor from chick ciliary ganglia. J. Neurosci. 10, 1711-1718.

Haim, N., Nahum, S. and Dudai, Y. (1979). Properties of a putative muscarinic cholinergic receptor from Drosophila melanogaster. J. Neurochem. 32, 543-552.

Hanke, W. and Breer H.(1986) Channel properties of a neuronal acetylcholine receptor protein purified from central nervous system of insect reconstituted in planar lipid bilayers. Nature (London) 321, 171-174.

Henrikson, R.I. and Meredith S. C. (1984) Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatizeation with phenylisothiocyanate. Analytical Biochem. 136, 65-74.

Hermans-Borgmeyer, I., Zopf, D., Ryseck, R.P., Hovemann, B., Betz, H. and Gundelfinger, E. (1986) Primary structure of a developmental regulated nicotinic acetylcholine receptor from Drosophila. EMBO J. 5, 1503-1508

Howard I.K., Sage H. J. and Stein M.D. (1971) Studies on a phytohemaglutinin from the lentil. J. Biol. Chem. 246, 1590-1595.

Hunkapiller, M.W., Lujian, E., Ostrander, F. and Hood, L.E. (1983) Isolation of microgram quantities of proteins from polyacrylamide gels. In Methods in Enzymology vol.91: 227-236.

Ikeda, K. (1980) Neuromuscular physiology. In "The genetics and Biology of Drosophila" (eds. Ashburner M. and Wright T.R.F.) Vol 2d, pp. 369-405, Academic Press, London, New York.

Jan, L.Y. and Jan, Y. N. (1976). L-glutamate as an excitatory transmitter at the Drosophila larva neuromuscular junction. J. Physiol. 262, 215-236.

Ji, T.H. (1977) A novel approach to the identification of surface receptors. J. Biol. Chem. 252, 1566-1570.

Ji, T.H. (1979) The application of chemical crosslinking for studies on cell membranes and the identification of surface receptors. Biochem. Biophys. Acta. 559, 39-69.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Levitan, E., Schofeld, P.R., Burt, D.R., Rhe, L.M., Wisden, W., Kohler, M., Fujita, N., Rodriquez, H.F., Stephenson, A., DArlison, M.G. Barnard, E.A. and Seeberg, P.H. (1988) Structural and functional basis for GABA receptor heterogeneity. Nature, 335, 76-79.

Lewis, E.B. (1960) A new standard food medium. Drosophila Inform. Serv. 34, 117-118.

Limbird, L.E. (1986) Cell surface receptors: A short course on the theory and methods . Martinus Nijhoff publishing.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Lummis, S. C. R. and Sattelle, D. B. (1985) Binding of N-[propionyl-^{3}H]-propionylated α-bungarotoxin and L-[benzilic-4,4'-^{3}H] quinuclidinyl benzilate to CNS extracts of the cockroach Periplaneta americana. Arch. Insect Biochem. Physiol. 3, 339-347.

Lunt G.G (1986) Is the insect neuronal nAChR the ancenstral ACh receptor protein? Trends. Neurosci. 341-342.

Maelicke, A. (1984) Nicotinic Acetylcholine Receptor Structure and Function , NATO ASI series, series H: Cell Biology, 3.

March, C.S., Cattell, K.J., and Donnellan, J.F. (1980) A simplified method for cyanogen bromide activation of agarose for affinity chromatography. Biochem. Soc. Trans. 8, 742-743.

Martin, R.G. and Ames, B.N. (1961) A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 236, 1372-1379

Maxwell, G. D., Tait, J. F. and Hildebrand, J. D. (1978) Regional synthesis of neurotransmitter candidates in the CNS of the moth Manduca sexta. Comp. Biochem. Physiol. 61C, 109-119.

Meeker, R.B., Michels, K.M.; Libber, M.T. and Hayward, J.N. (1986) Characteristics and distribution of high- and low-affinity alpha bungarotoxin binding sites in rat hypothalamus. J. Neurosci. 6, 1866-1875.

Meunier, J.-C.,Olsen, R.W. and Changeux, J.-P. (1972) Studies on the cholinergic receptor protein from electrophorus electrics, effect of detergents on some hydrodynamic properties of the receptor protein in solution. FEBS Lett. 24, 63-68.

Peterson, G.L. (1983) in Methods in Enzymology (Hirs, C.H.W., and Timasheff, S.N. eds) Vol. 91, pp.95-119, Academic Press, New York

Prescott D. J., Hildebrand J. G., Sanes J. R. and Jewett S. (1977) Biochemical and developmental studies of acetylcholine metabolism in the central nervous system of the moth Manduca sexta. Comp. Biochem. physiol. 56C, 77-84.

Restifo, L.L. and White, K. (1989) Molecular and genetic approaches to neurotransmitter and neuromodulator systems in Drosophila Adv. Insect Physiol. 22.

Rudloff, E. (1978) Acetylcholine receptors in the central nervous system of Drosophila melanogaster. Exp. Cell Res. 111, 185-190.

Salinovich, O.and Montelaro, R.C. (1986) Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Anal. Biochem. 156, 341-347.

Sambook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning A laborotory manual. Cold Spring Harbor Laborotory Press.

Sattelle, D.B. (1980) Acetylcholine receptors of insects. Adv. Insect Physiol. 15, 215-315.

Sawruk, E., Schloβ, P., Betz, H., and Schmitt, B. (1990) Heterogeneity of Drosophila nicotinic acetylcholine receptors: SAD, a novel developmentally regulated α-subunit. EMBO J. 9, 2671-2677, 1990.

Schloβ, P., Hermans-Borgmeyer, I., Betz, H., and Gundelfinger, E.D. (1988) Neuronal acetylcholine receptors in Drosophila: the ARD protein is a component of a high-affinity α-bungarotoxin binding complex. EMBO J. 7, 2889-2894

Schmidt-Glenewinkel, T., Venkatesh, T.R. and Hall, L.M. (1980) Purification and characterization of a nicotinic acetylcholine receptor from Drosophila melanogaster. 10th Meeting Soc. Neurosci. (Cincinnati) Abst.

Schmidt-Nielsen, B.K., Gepner, J.I., Teng, N.N.H., and Hall, L.M. (1977) Characterization of an α-bungarotoxin binding component from Drosophila melanogaster J. Neurochem. 29, 1013-1029.

Sharp, P. M., Cowe, E., Higgins, D. G., Shields, D.C., Wolfe, K.H. and Wright, F. (1988) Codon usage patterns in Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Drosophila melanogaster and Homo sapiens; a review of the considerable within-species diversity. Nucleic Acids Res. 16, 8207-8211.

Shimohama, S. Taniguchi, T., Fujiware, M. and Kameyama, M.(1986) Changes in nicotinic and muscarinic cholinergic receptors in Alzheimer-type dementia. J. Neurochem 46, 288-293.

Siegel, L.M. and Monley, K.J. (1966) Determination of molecular weights and fractional ratios of proteins in impure systems by use gel filtration and density gradient centrifugation, application to crude preparations of sulfite and hydroxylamine reductase. Biochim. Biophys. Acta. 112. 346-362.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76, 4350-4354.

Tu, A.T. (1973) Neurotoxins of animal venoms: snakes. Ann. Rev. Biochem. 42, 235-258.

Wessel, D. and Flugge, U.I. (1984) A method for the quantitativy recovery of protein in dilute solution in the presence of detergents and lipids. Analytic Biochem. 138, 141-143.

Whiting, P., and Lindstrom, J. (1986) Purification and characterization of a nicotinic acetylcholine receptor from chick brain. Biochemistry 25, 2082-2093

Whiting, P., and Lindstrom, J. (1987) Puriication and characterization of a nicotinic acetylcholine receptor from rat brain. Proc. Natl. Acad. Sci. (USA) 84, 595-599

Yuen, .W., Chui, A.H. , Wilson, K.J. and Yuan P.M. (1988) Microanalysis of SDS-PAGE electrobloted proteins. User Bulletin, issue no. 36. Applied Biosystems,Foster City Biosystem Division.