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Activation of Botulinum C_2 Toxin by Trypsin

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C_2 toxin (C2T) elaborated by *Clostridium botulinum* types C and D is composed of two dissimilar protein components, designated components I and II. The biological activity of C2T is enhanced by treating the toxin with trypsin. This activation of C2T is observed as a result of mixing untrypsinized component I and trypsinized component II but not as a result of mixing trypsinized component I and untrypsinized component II. The data presented here show that the maximum lethality of C2T, determined by mixing untrypsinized component I and trypsinized component II, was attained by treating component II with trypsin at a ratio of 10:1 on a protein basis for 30 min at 35°C at pH 7.5. The activation of component II was always accompanied by a change in the molecular weight of the component from 101,000 to 88,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). However, the gel filtration of trypsinized component II resulted in the separation of two active components, with apparent molecular weights, estimated from the elution volume by gel filtration, of 365,000 and 74,000. The high-molecular-weight component II had hemagglutination and hemolytic activities, whereas the low-molecular-weight component II has only hemagglutination activity. These two molecular species of active component II had approximately the same lethality, when mixed with component I, and gave a single band in SDS-PAGE, with a molecular weight of 88,000, the same as that of trypsin-activated component II under different reaction conditions. The results indicate that the activation of C2T by trypsin is due to the molecular conversion of component II from molecular weight 101,000 to 88,000 as determined by SDS-PAGE and that the trypsin-activated component II tends to form an oligomer of the active component II.

C_2 toxin (C2T), elaborated by most *Clostridium botulinum* type C and D strains, is composed of two separate protein components, designated components I and II, with molecular weights of 55,000 and 105,000, respectively, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15). These two components are not linked, either with covalent or noncovalent bonds (15). The components act together as dual-function molecules; component II recognizes the receptor site on the cell surface membrane, and component I is the effector in the cytoplasm (12, 14, 17). C2T, as well as being lethal, has novel biological activities, including enterotoxic, cytotoxic, and vascular permeability activities (13,, 16, 18). All of these activities are enhanced by treating the toxin, a mixture of components I and II, with trypsin. Previous studies have shown that the full activity of C2T is attained by a mixture of untrypsinized component I and trypsinized component II, but not of trypsinized component I and untrypsinized component II, indicating that the activation of C2T is due to the molecular cleavage of component II by trypsin and that the activation of C2T is equivalent to the activation of component II by trypsin (13, 15, 16). The present study, therefore, was initiated to investigate the changes in molecular size of component II during trypsinization in relation to the enhancement of the lethality of C2T. In addition, active component II after trypsinization was separated by gel filtration and characterized.

MATERIALS AND METHODS

Materials. Trypsin (type III, twice crystallized), chymotrypsin (type II), papain (type IV), bromelain (grade II), and chymotrypsinogen A were purchased from Sigma Chemical Co., St. Louis, Mo. Urease, catalase, aldolase, bovine serum albumin, ovalbumin, β-galactosidase, and phosphorylase *a* were from Boehringer GmbH, Mannheim, Federal Republic of Germany. Alcohol dehydrogenase was from Miles Seravac, Maidenhead, England. Soybean trypsin inhibitor was from Worthington Diagnostics, Freehold, N.J. Sephadex G-100 was from Pharmacia, Uppsala, Sweden, and Bio-Gel P-300 was from Bio-Rad Laboratories, Richmond, Calif.

Preparation of two components of C2T. Components I and II were purified from the culture fluid of *C. botulinum* type C strain 92-13 as described previously (15).

Determination of toxicity. The intraperitoneal 50% lethal dose ($ipLD_{50}$) of C2T was determined by the time-to-death method; the toxin sample was injected intravenously in 0.1-ml volumes into three mice, and the average time to death (in minutes) was used to calculate the $ipLD_{50}$ from the standard plot of the time-to-death versus $ipLD_{50}$ (15). The C2T in the present study was defined as a mixture of component II and component I at a 2:1 ratio on a protein basis.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 7.5% polyacrylamide gel by the method of Weber et al. (24). A sample was mixed with an equal volume of a solution containing 2% mercaptoethanol, 8 M urea, and 2% SDS. The mixture was heated for 3 min in a boiling-water bath, and then a 50-μl portion of the mixture, containing 5 to 15 μg of protein, was applied to a gel column (0.6 by 8 cm). Electrophoresis was carried out at 6 mA per column for 180 to 210 min. After electrophoresis, the gel columns were stained with 0.1% Coomassie brilliant blue in 50% trichroloacetic acid for 10 min at 40°C and destained with 7% acetic acid at room temperature. To determine the molecular weight (M_r), I used the following protein standards: β-galactosidase (M_r, 130,000), phosphorylase *a* (M_r, 94,000), bovine serum albumin (M_r, 65,000), ovalbumin (M_r, 45,000) and chymotrypsinogen A (M_r, 25,000).

Determination of molecular weight by gel filtration. The Sephadex G-100 column (2.5 by 95 cm) was equilibrated and

TABLE 1. Activation of component II by proteases[a]

Protease	Lethality (ipLD$_{50}$/ml of reaction mixture)
None[b]	S[c]
Trypsin	66.8
Bromelain	S[c]
Papain	0.26
Chymotrypsin	23.5
Chymotrypsin + STI[d]	S[c]

[a] Component II (20 μg) was reacted in 980 μl of 50 mM potassium-sodium phosphate buffer (pH 8.0) with trypsin (20 μg), 50 mM potassium-sodium phosphate buffer–5 mM cysteine hydrochloride (pH 7.5) with bromelain (20 μg), 50 mM Tris-hydrochloride–5 mM cysteine hydrochloride–2 mM EDTA (pH 8.0), with papain (20 μg), and 50 mM Tris-hydrochloride (pH 8.0) with chymotrypsin (20 μg). After incubation at 35°C for 30 min, the reaction mixtures were mixed with 20 μl of component I (500 μg/ml), and the lethality was determined as described in Materials and Methods.

[b] Reaction conditions were the same for tryspin, except that the enzyme was omitted.

[c] Mice survived for 3 h or more.

[d] Soybean trypsin inhibitor (STI; 20 μg) was added to the reaction mixture.

eluted at 4°C with 0.2 M NaCl–50 mM potassium-sodium phosphate buffer (pH 7.5). The molecular weight was determined by the method of Andrews (2) by using the following standard proteins; urease (M_r, 483,000), catalase (M_r, 240,000), aldolase (M_r, 158,000), and bovine serum albumin (M_r, 65,000).

Assay for trypsin activity. The amidase activity of trypsin was assayed by the method of Erlanger et al. (6). The reaction mixture of 1.35 ml consisted of 1.25 ml of 1 mM substrate (*N*-benzoyl-DL-arginine *p*-nitroanilide) in 0.1 M Tris hydrochloride (pH 7.5), and 0.1 ml of an enzyme solution. The reaction mixtures were incubated at 35°C for 30 min, and 0.25 ml of 30% acetic acid was added to stop the reaction. *p*-Nitroanilide released was measured spectrophotometrically at 410 nm. The activity was expressed in an arbitrary unit defined as the activity liberating 1 nmol of *p*-nitroanilide per min under the specific conditions.

Assay for hemagglutination and hemolytic activities. Heparinized mouse blood was centrifuged. The erythrocytes were washed twice with 0.15 M NaCl–10 mM potassium-sodium phosphate buffer (pH 7.3) (PBS) and suspended in PBS at 0.05% (vol/vol). Samples (50 μl) were diluted twofold serially in PBS in a 98-well U-shaped plastic plate and mixed with an equal volume of the mouse erythrocyte suspension. The plate was incubated for 2 h at 25°C. The hemagglutination and hemolysis of component II could be read on the same lane of the sample, because the hemagglutination was always higher than the hemolysis. One hemagglutination unit was the reciprocal of the highest dilution which agglutinated the cells completely. Likewise, one hemolytic unit was the reciprocal of the highest dilution which lysed the cells completely.

Protein determination. Protein was determined by the method of Lowry et al. (10).

RESULTS

Activation of component II by proteases. Component II was treated with several proteases, including trypsin, and its lethality was determined by mixing it with component I to see whether the activation of C2T is caused by treating component II with proteases other than trypsin. The increase in lethality of C2T occurred as a result of treating component II with chymotrypsin and trypsin (Table 1). However, this increase in the lethality of C2T as a result of chymotrypsin addition seems to be due to trypsin contaminated in the enzyme preparation, because the increase was

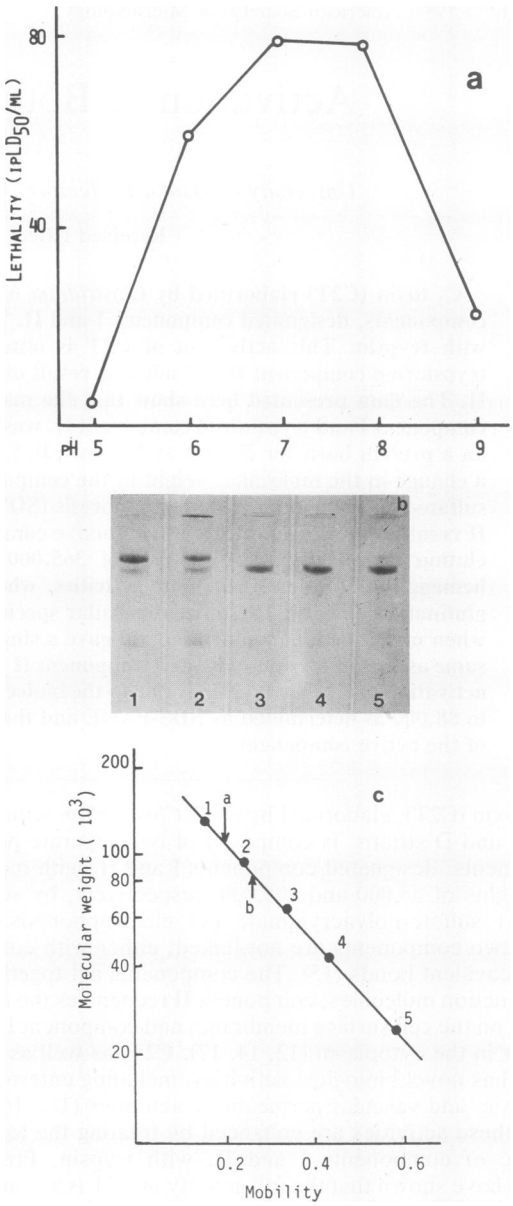

FIG. 1. Activation of component II by trypsin at different pHs. Component II (50 μg) was incubated for 30 min at 35°C with trypsin (5 μg) in 100 μl of 0.1 M acetate buffer (pH 5.0 and pH 6.0), 0.075 M potassium-sodium phosphate buffer (pH 7.0 and 8.0), and 0.1 M $NaHCO_3$–NaOH (pH 9.0). At the end of the incubation, two 30-μl portions were removed from each reaction mixture. (a) One portion was mixed with 30 μl of component I (250 μg/ml), diluted to 1.0 ml with ice-cold PBS, and injected into mice to determine the lethality. (b) The other portion was used for SDS-PAGE. Lanes: 1, pH 5.0; 2, pH 6.0; 3, pH 7.0; 4, pH 8.0; 5, pH 9.0. (c) Determination of molecular weight of trypsin-activated component II. Standard proteins: 1, β-galactosidase; 2, phosphorylase *a*; 3, bovine serum albumin; 4, ovalbumin; 5, chymotrypsinogen A. Arrows a and b indicate untrypsinized and trypsinized component II, respectively. For details, see Materials and Methods.

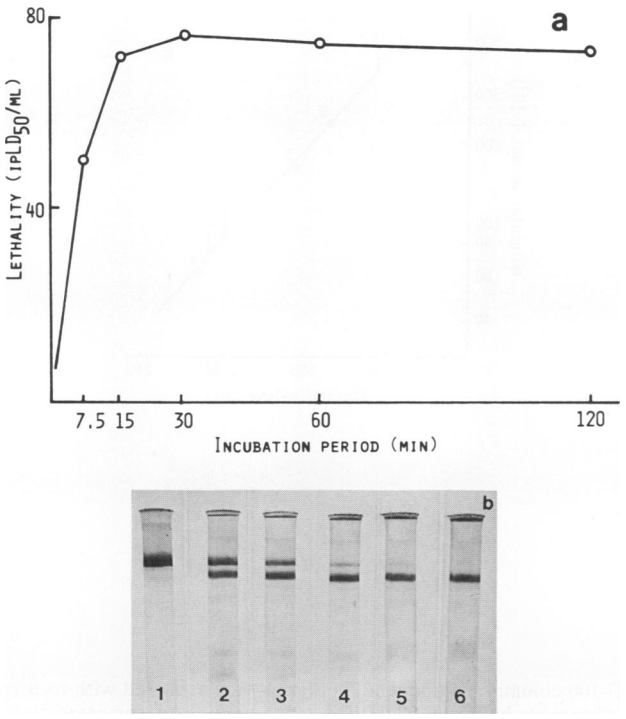

FIG. 2. Time course of activation of component II by trypsin. Component II (500 μg) was incubated at 35°C with trypsin (20 μg) in 1 ml of 0.1 M Tris hydrochloride (pH 7.5). Two 30-μl portions of the reaction mixtures were removed at the time indicated. (a) Determination of lethality. (b) SDS-PAGE. Lanes: 1, intact component II; 2, 7.5-min incubation; 3, 15 min; 4, 30 min; 5, 60 min; 6, 120 min incubation. Determination of lethality and SDS-PAGE were carried out as described in the legend to Fig. 1.

eliminated when soybean trypsin inhibitor was added to the reaction mixture. In the following experiments, therefore, trypsin was used for the activation of component II.

Conditions of activation of component II by trypsin. (i) Effect of pH. Component II was trypsinized at different pH values, and the lethality was determined by mixing it with component I. In addition, the product(s) of trypsinized component II was analyzed by SDS-PAGE to examine the molecular cleavage of component II by trypsin. The optimum pH for activation of component II by trypsin was between 7.0 and 8.0 (Fig. 1a). SDS-PAGE patterns show that trypsin activation of component II at pH 7.0 and 8.0 resulted in a complete conversion of component II to a lower molecular weight than that of untrypsinized component II (Fig. 1b). The molecular weights of untrypsinized and trypsinized component II determined by SDS-PAGE were 101,000 and 88,000, respectively (Fig. 1c), which were the same before and after the components were treated with a reducing agent (data were not shown), indicating that the trypsin-activated component II was not a nicked form, as the botulinum neurotoxins are (4).

(ii) Effect of incubation period. The increase in the toxicity of C2T and the change in molecular weight of component II as a result of trypsin treatment were investigated as a function of time. The maximum activity was attained after 30-min of incubation at 35°C and remained at almost the same level for 120 min (Fig. 2a). SDS-PAGE patterns show that the molecular weight conversion of component II from 101,000 to 88,000 during trypsinization proceeded parallel with the increase in the toxicity of C2T, and an almost complete conversion of component II at a trypsin-to-component-II ratio at 1:25 on a protein basis occurred after 60 min of incubation (Fig. 2b). In SDS-PAGE, some insoluble proteins were observed on the top of the gels when component II was trypsinized at the protein concentration used.

(iii) Effect of trypsin concentration. The maximum activation of component II was attained at a trypsin-to-component II ratio at 1:8 on a protein basis (Fig. 3a). A higher concentration of trypsin caused the degradation of component II and a decrease in the lethality of C2T, while a lower concentration of trypsin caused incomplete conversion of component II from molecular weight 101,000 to 88,000 and also resulted in lower lethality (Fig. 3b).

The results described above show that the optimum conditions for activation of component II by trypsin are the incubation of component II with trypsin at a ratio of 10:1 on a protein basis for 30 min at 35°C either in 0.1 M Tris hydrochloride (pH 7.5) or in 50 mM potassium-sodium phosphate buffer (pH 7.5).

Separation of active component II by gel filtration on a Sephadex G-100 column. To separate the active component II, the trypsin-activated component II was gel filtered through a Sephadex G-100 column. The active form of component II was resolved into two separate peaks by the gel filtration (Fig. 4a).The apparent molecular weights of these two proteins, determined by gel filtration, were 365,000 and 74,000; the molecular weight of untrypsinized component II by gel filtration on the same column was

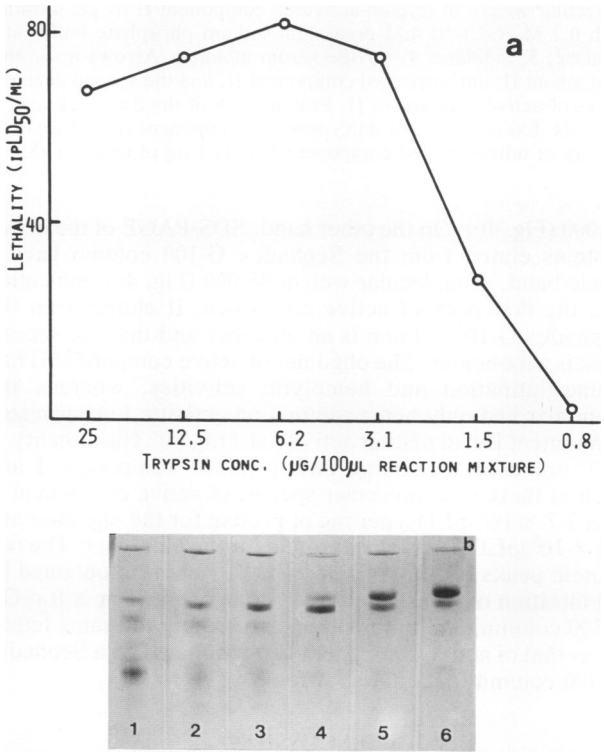

FIG. 3. Activation of component II at different trypsin concentrations. Component II (50 μg) was incubated for 30 min at 35°C with trypsin at the concentrations indicated in 100 μl of 0.1 M Tris hydrochloride (pH 7.5). (a) Determination of lethality. (b) SDS-PAGE. Lanes: 1, 25 μg of trypsin per ml of reaction mixture; 2, 12.5 μg; 3, 6.2 μg; 4, 3.1 μg; 5, 1.5 μg; 6, 0.8 μg. Determination of lethality and SDS-PAGE were carried out as described in the legend to Fig. 1.

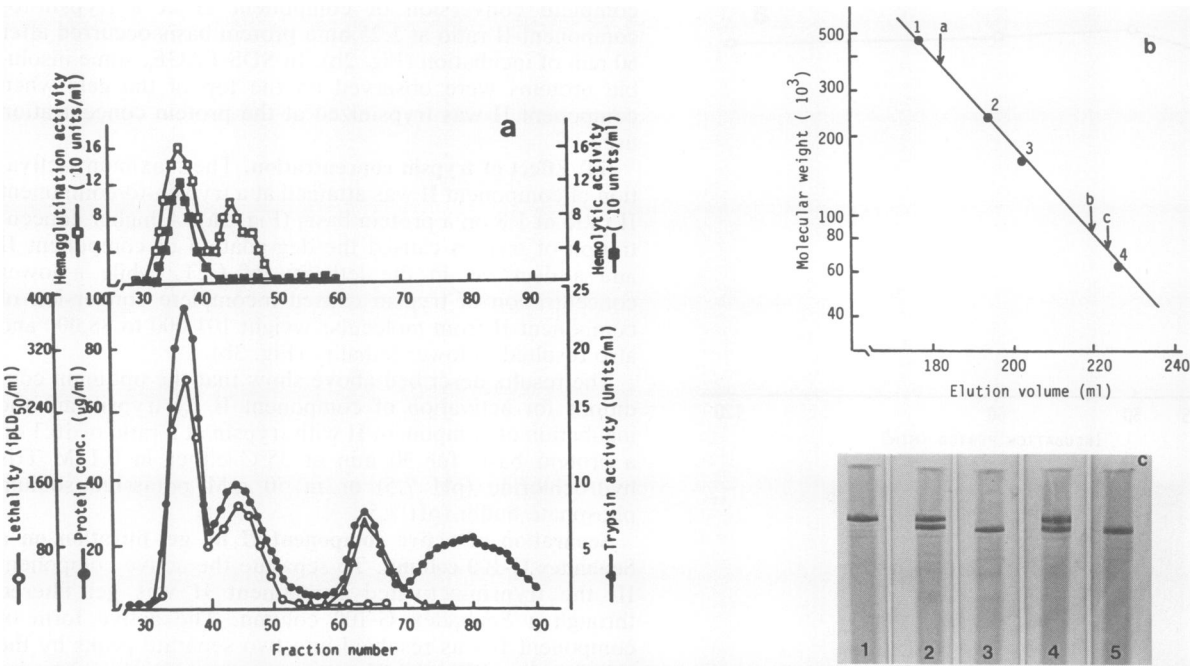

FIG. 4. Gel filtration of trypsin-activated component II on a Sephadex G-100 column. Component II (10 mg) was incubated with trypsin (1 mg) for 30 min at 35°C in 10 ml of 0.2 M NaCl–50 mM potassium-sodium phosphate buffer (pH 7.5). The reaction mixture was centrifuged at 10,000 × *g* for 20 min to remove the insoluble proteins accumulated during trypsinization, and the supernatant was applied to a Sephadex G-100 column (2.5 by 95 cm), which was equilibrated with 0.2 M NaCl–50 mM potassium-sodium phosphate buffer (pH 7.5) and eluted with the same buffer at 4°C. (a) Elution patterns of protein, biological activities of component II, and trypsin activity. (b) Estimation of the molecular weight of trypsin-activated component II by gel filtration on a Sephadex G-100 column. The column (2.5 by 95 cm), equilibrated with 0.2 M NaCl–50 mM potassium-sodium phosphate buffer at 4°C, was calibrated with the following standards proteins: 1, urease; 2, catalase; 3, aldolase; 4, bovine serum albumin. Arrows a, b, and c indicate the elution positions of the first protein peak of trypsinized component II, untrypsinized component II, and the second peak of trypsinized component II, respectively. (c) SDS-PAGE of the two protein peaks of active component II. Fractions 36 of the first peak and 45 of the second peak were concentrated and used for the electrophoretic analysis. Lanes: 1, 5 μ of untrypsinized component II; 2, 5 μg of untrypsinized component II plus 5 μg of fraction 36; 3, 5 μg of fraction 36; 4, 5 μg of untrypsinized component II plus 5 μg of fraction 45; 5, 5 μg of fraction 45.

84,000 (Fig. 4b). On the other hand, SDS-PAGE of these two proteins eluted from the Sephadex G-100 column gave a single band, of molecular weight 88,000 (Fig. 4c), indicating that the first peak of active component II eluted from the Sephadex G-100 column is an oligomer and that the second peak is a monomer. The oligomer of active component II had hemagglutination and hemolytic activities, whereas the monomer had only hemagglutination activity; untrypsinized component II had neither activity at 1 mg/ml. The lethality of C2T prepared by mixing untrypsinized component I and each of these two molecular species of active component II was 3.7×10^3 $ipLD_{50}$ per mg of protein for the oligomer and 3.1×10^3 $ipLD_{50}$ per mg of protein for the monomer. The two protein peaks of active component II were also obtained by gel filtration of trypsin-activated component II on a Bio-Gel P-300 column, and both had approximately the same lethality as that of active component II fractionated on a Sephadex G-100 column (data not shown).

DISCUSSION

The evidence presented here shows that the activation of component II by trypsin, as observed by an increase in the lethality of C2T, always accompanies the change in the molecular weight of component II from 101,000 to 88,000 in SDS-PAGE. During the activation, neither an intermediate nor a nicked form of component II was found. These results indicate that the activation of component II by trypsin is due to molecular cleavage of the component at a single internal peptide bond. Previous studies have shown that the trypsin-treated component II binds to the cell surface membrane and induces the binding site for component I, whereas the intact component II binds to the membrane but does not induce the site for component I (17). These facts indicate that the ability to bind to the cell membrane is a native property for component II, but that induction of the binding site for component I is generated by the removal of a small peptide from the component II molecule by trypsin.

The gel filtration of trypsin-activated component II on a Sephadex G-100 column resolved the active component II into two separate fractions. This does not seem to be due to an interaction of component II with the Sephadex gel matrix, which is cross-linked dextran, because a similar pattern was obtained by gel filtration of trypsin-activated component II on a column of Bio-Gel P-300, which is cross-linked polyacrylamide gel. The apparent molecular weights of the two active forms of component II estimated by gel filtration on a Sephadex G-100 column were 365,000 and 74,000, whereas those determined by SDS-PAGE were both 88,000, which is consistent with the values obtained by trypsinization of component II under various conditions. These results show that trypsin-activated component II tends to form an oligomer consisting of M_r 74,000 monomers.

The molecular species of the oligomer and the monomer separated by gel filtration had approximately the same lethality on a protein basis, suggesting that the oligomer of

active component II acts as a monomer in vivo. However, this does not exclude the possibility that active component II forms an oligomer on the cell surface membrane, where the binding of component II to the membrane induces the binding site for component I.

Some plant and bacterial protein toxins consist of two functionally distinct parts, an A component that is enzymatically active and a B component that binds to the cell surface receptor (7). These include abrin (20), ricin (3, 11), cholera toxin (8), diphtheria toxin (21), and pertussis toxin (23). The B components of these toxins have lectinlike activity, which is characterized by agglutination of erythrocytes (3, 11, 20), binding to a carbohydrate-containing molecule on the cell surface membrane (9, 11), or competitive inhibition of the binding of the toxin to cell membranes by lectin, glycoprotein, or mono- and oligosaccharides (3, 5, 11, 22). The present study demonstrates that trypsin-activated component II separated by gel filtration, both monomer and oligomer forms, had hemagglutination activity, whereas intact component II did not. These observations suggest that the active component II of C2T may recognize and bind to the carbohydrate-containing molecule on the cell surface membrane, which possibly differs from the binding site of intact component II. Recent studies have shown that the binding site for component I of C2T to the cell membrane is induced by trypsin-activated component II and that component I catalyzes the transfer of the ADP-ribose moiety of NAD to cytoplasmic actin (1, 14, 19). Thus, components I and II of C2T correspond to the A and B components of A-B model toxin, respectively, which consists of the enzymatically active component and the binding component (7).

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