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PREPARATION OF PURE PEPTIDES FROM A MIXTURE OF GRAMICIDINS AND OF TYROCIDINES BY DROPLET COUNTERCURRENT CHROMATOGRAPHY

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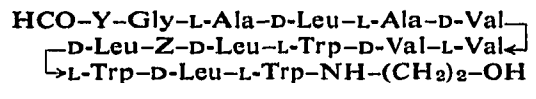
SUMMARY

Crude gramicidin was fractionated into the pure components, gramicidins A, B and C, by droplet countercurrent chromatography. As crude tyrocidine contains a small amount of a gramicidin-like peptide, the material was treated previously by droplet countercurrent chromatography to yield a partially purified tyrocidine. This was fractionated into pure tyrocidines A, B and C. Melting point, specific rotation, antibacterial activity, ultraviolet absorption curve and optical rotatory dispersion data are presented for the pure peptides obtained.

INTRODUCTION

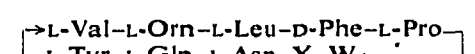
Gregory and Craig fractionated a commercial gramicidin into three pure components by countercurrent distribution, and designated them as gramicidin (Gdin) A, Gdin B, and Gdin C (ref. 1). Gross and Witkop extended this original method and isolated Gdin D as an additional minor component[2]. Sarges and Witkop elucidated the structure of these components as shown in Fig. 1 (refs. 3–5). Craig and his collaborators also fractionated crude tyrocidine into tyrocidine (Tdin) A (ref. 6), Tdin B (ref. 7) and Tdin C (ref. 8) by countercurrent distribution. Their structures were elucidated as shown in Fig. 2 (refs. 8–10).

A new technique of countercurrent chromatography, namely droplet countercurrent chromatography (DCCC), has been introduced by Tanimura *et al.*[11], who demonstrated that a mixture of dinitrophenylamino acids was separated efficiently by DCCC[11]. Nakayama *et al.* obtained pure fowl angiotensin from a partially purified peptide by DCCC[12].



	(Y)	(Z)
[Val^1]-Gdin A	L-Ile	L-Trp
[Ile^1]-Gdin A	L-Ile	L-Trp
[Val^1]-Gdin B	L-Val	L-Phe
[Ile^1]-Gdin B	L-Ile	L-Phe
[Val^1]-Gdin C	L-Val	L-Tyr
[Ile^1]-Gdin C	L-Ile	L-Tyr

Fig. 1. Structure of the gramicidin (Gdin) family.



	(W)	(X)
Tdin A	L-Phe	D-Phe
Tdin B	L-Trp	D-Phe
Tdin C	L-Trp	D-Trp

Fig. 2. Structure of the tyrocidine (Tdin) family.

This paper describes the preparation of pure components from crude gramicidin and tyrocidine by the use of DCCC, as part of a study of the chemical synthesis of peptides corresponding to the sequences of natural gramicidin and tyrocidine[13–16]. DCCC seems particularly suitable for purification of synthetic crude peptides as it appeared well suited to the separation of mixtures with similar structures. We also wanted to describe some physical constants, such as specific rotation, for all pure natural peptides as descriptions in the literature are incomplete.

EXPERIMENTAL

Materials

Crude natural Gdin (S. B. Penick & Co., Lot No. 640) and Tdin (Rapidase, Lot No. 29302) were donated by Dr. E. Gross (Nat. Inst. Health, Bethesda, Md., U.S.A.) and Dr. C. R. Pollet (Rapidase, Nord, France), respectively. It was found that crude Tdin is composed of tyrocidines (85%) and gramicidin-like peptides (15%). Therefore, partially purified tyrocidine hydrochloride (PP-Tdin·HCl), which is a starting material for the preparation of pure components, was prepared as follows. The crude Tdin (200 mg) was subjected to DCCC by the procedure described in a later section. The chromatogram is shown in Fig. 3, and fractions 228–300 were evaporated *in vacuo* to dryness to yield 173 mg of residual white powder (PP-Tdin·HCl).

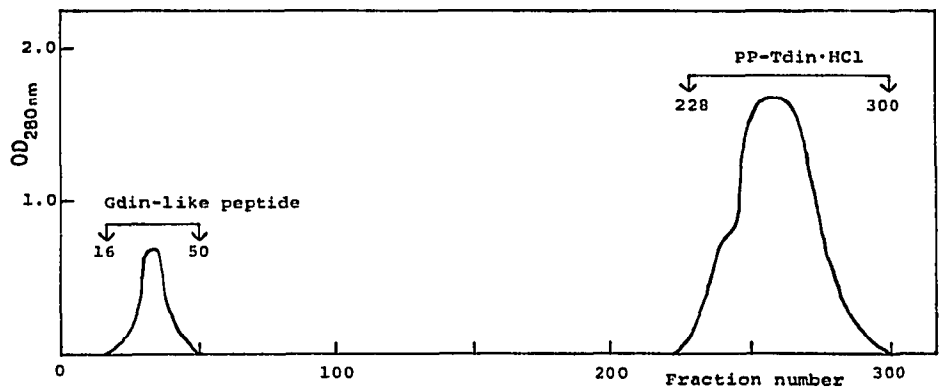


Fig. 3. Preparation of PP-Tdin·HCl by the droplet countercurrent distribution of crude Tdin. Column No., 100; solvent, S_1. One fraction is 4 g.

Droplet countercurrent chromatography

An apparatus made by Seikagaku Kogyo (Tokyo, Japan) was used. It consists of 300 column units (mounted perpendicularly) of glass tubing (0.6 mm wall thickness and 2.4 mm I.D.) 60 cm long and connected by PTFE tubing (0.5 mm I.D.). The solvents used were as follows: S_1 = benzene–chloroform–methanol–0.1 *N*

HCl (11:5:10:4); S_2 = benzene–chloroform–methanol–water (15:15:23:7); S_3 = chloroform–methanol–0.1 *N* HCl (19:19:12). A solvent mixture was allowed to equilibrate in a funnel and the upper phase was then loaded into the required units of the glass columns as stationary phase. A sample dissolved in the lower phase (3 ml) was placed at the top of the first column, and the lower phase in the funnel was pumped as mobile phase by nitrogen pressure through the top of this column at a flow-rate of 12–15 ml/h. All experiments were carried out at room temperature. Fractions from the last column were collected, and their absorbances were determined at 280 nm.

Amino acid analysis

A sample (1.5 mg) was hydrolysed with 6 *N* HCl either with 4% thioglycolic acid[17] (for tryptophan-containing peptide) or without thioglycolic acid for 48 h in an evacuated, sealed tube at 110°, and was recorded on a Hitachi (Mt. View, Calif., U.S.A.) Model KLA-3A amino acid analyser.

Ultraviolet absorption and optical rotatory dispersion

The UV absorption of a sample in methanol at room temperature was recorded on a Hitachi Model 124 spectrophotometer. The optical rotatory dispersion (ORD) curve was measured by a Jasco Model ORD-CD/UV-5 spectropolarimeter under the following conditions: wavelength range, 210–300 nm; concentration, 1 mg/ml in ethanol; cell pathlength, 0.02 cm.

Microbiological assay

The minimum inhibitory concentration was determined by a dilution method using a nutrient agar and a synthetic agar. Gdin S was examined as a reference compound.

RESULTS AND DISCUSSION

Attempted separation by Sephadex LH-20

Several investigators have succeeded in the separation of a mixture of lipophilic peptide derivatives on Sephadex LH-20, *e.g.* of a diastereomeric mixture of benzyloxycarbonyl-L-Leu–DL-Val[18]. Crude Gdin and PP-Tdin·HCl (each 5 mg)were therefore chromatographed on a Sephadex LH-20 column. Absorbance at 280 nm of the effluent was measured by a continuous-flow UV monitor (Uvicon-540; Toyo Roshi, Tokyo, Japan). Crude Gdin or PP-Tdin, which consists of at least three components, was not separated completely (Fig. 4)

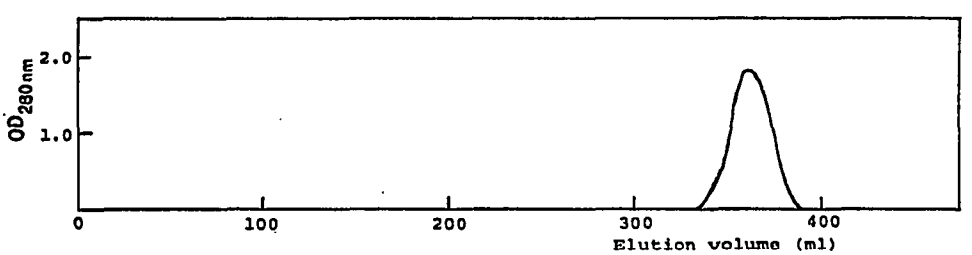


Fig. 4. Chromatography on a 2.8 × 120 cm column of Sephadex LH-20 of crude Gdin. Solvent, methanol; flow-rate, 40 ml/h.

Preparation of Gdin A, B, and C

Crude Gdin (53.3 mg) dissolved in the lower phase of S_2 was subjected to DCCC, and the moving phase was collected until fraction number 155. After number 156, the stationary phase was ejected from the last column by nitrogen pressure. As shown in Fig. 5, crude Gdin was completely fractionated into three major components. The three fractions (numbers 30–60, 70–130 and 157–185) were evaporated *in vacuo*, and the residual powders were collected by filtration with the aid of water. These peptides were identified as Gdin B, A, and C by amino acid analysis. Fig. 5 shows that the highly lipophilic Gdin B and A are more soluble in the lower phase of the solvent, while less lipophilic Gdin C, in which one tryptophan residue is replaced by tyrosine (Fig. 1), is soluble in the upper phase. Table I gives the yield of each component. It should be noted that the recovery was as high as 95%.

Table I gives also melting point and specific rotation data for the peptides obtained. Melting point data of natural Gdin A and B (ref. 1), and specific rotation data of Gdin A (ref. 3) have been reported.

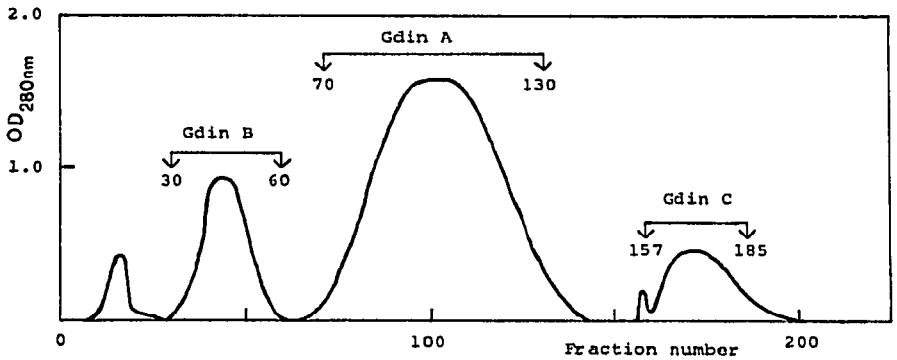


Fig. 5. Droplet countercurrent chromatogram of crude Gdin. Column No., 200; solvent, S_2. One fraction is 6 g.

TABLE I

YIELDS AND SOME PROPERTIES OF THE PURIFIED GRAMICIDINS

Compound	*Yield**		*Total yield*		*Mp.* (°C)	$[\alpha]_D^{25}$
	mg	%	*mg*	%		
Gdin B	9.8	18	51	95	245–247***	+6.0° (*c* 0.72, DMF)**
Gdin A	35.7	67			221–224§	+5.1° (*c* 0.86, DMF)§§
Gdin C	6.5	12			280–283 (decomp.)	+11.4° (*c* 0.29, DMF)

* Crude Gdin (53.3 mg) was subjected to DCCC (see Fig. 5).
** DMF = Dimethylformamide.
*** Reported value, 258–259° (ref. 1).
§ Reported value, 227–228° (ref. 1).
§§ Reported value, +5.0° (DMF) (ref. 3).

Sequential analysis[3] of Gdin A has established that pure Gdin A obtained by countercurrent chromatography consists of two analogues, namely [Val^1]-Gdin A (major component) and [Ile^1]-Gdin A (minor component). It was also found that pure Gdin B and C are a mixture of [Val^1]- and [Ile^1]-analogues, respectively[4,5]. In this study, the amino acid analyses (Table II) of three peptides (Gdin A, B, and C) show the presence of two analogues in each peptide. To examine the distribution of [Val^1]- and [Ile^1]-Gdin A in a peak corresponding to Gdin A in Fig. 5, the peak was divided into three parts as noted in Table III. A small amount of each part was subjected to amino acid analysis and the ratio of the two analogues was calculated as shown in Table III. It was observed that the more lipophilic [Ile^1]-Gdin A is more soluble in the lower phase of the solvent than [Val^1]-Gdin A. The result also suggests that each analogue might be isolated by increasing the number of units of glass columns in DCCC.

TABLE II

AMINO ACID COMPOSITIONS OF THE PURIFIED GRAMICIDINS

Numbers in parentheses give the theoretical amino acid composition.

Amino acid	*Gdin B*	*Gdin A*	*Gdin C*
Gly	0.99 (1)	1.02 (1)	1.09 (1)
Ala	2.00 (2)	2.00 (2)	2.00 (2)
Val	3.78 (~4)	4.06 (~4)	3.99 (~4)
Ile	0.13 (~0)	0.13 (~0)	0.06 (~0)
Leu	4.04 (4)	4.05 (4)	4.12 (4)
Phe	0.93 (1)	0.00 (0)	0.00 (0)
Tyr	0.00 (0)	0.00 (0)	0.87 (1)
Trp	2.86 (3)	3.75 (4)	2.78 (3)

TABLE III

COMPOSITION OF TWO ANALOGUES IN DIVIDED FRACTIONS OF GRAMICIDIN A IN FIG. 5

Compound	*Ratio of two analogues (%)*			
	(Total fraction 70–130)	*Fraction 70–85*	*Fraction 86–115*	*Fraction 116–130*
[Val^1]-Gdin A	(76)	41	92	96
[Ile^1]-Gdin A	(24)	59	8	4

Preparation of Tdin A, B, and C

PP-Tdin·HCl (50 mg) was purified by DCCC (see Fig. 6). Three fractions (numbers 20–35, 45–75 and 90–120) were treated as described for the preparation of Gdin A, B, and C. The three products isolated were designated as partially purified tyrocidin A hydrochloride (PP-Tdin A·HCl), PP-Tdin B·HCl, and PP-Tdin C·HCl, yields of the products being shown in Table IV. To obtain pure

peptides, each product was re-chromatographed by the above procedure (see Fig. 7). The main peak in each re-chromatography (*e.g.*, fractions 10–35 in Fig. 7a) yielded a pure peptide, yields being shown in Table IV. The melting points and

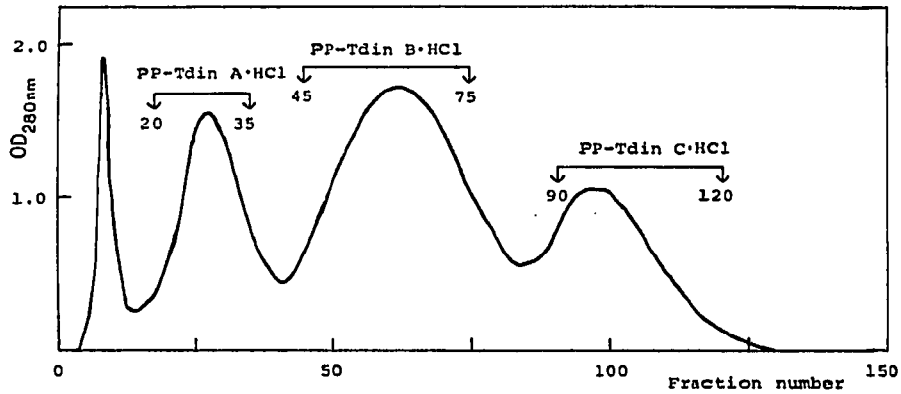


Fig. 6. Droplet countercurrent chromatogram of PP-Tdin·HCl. Column No., 150; solvent S_3. One fraction is 4 g.

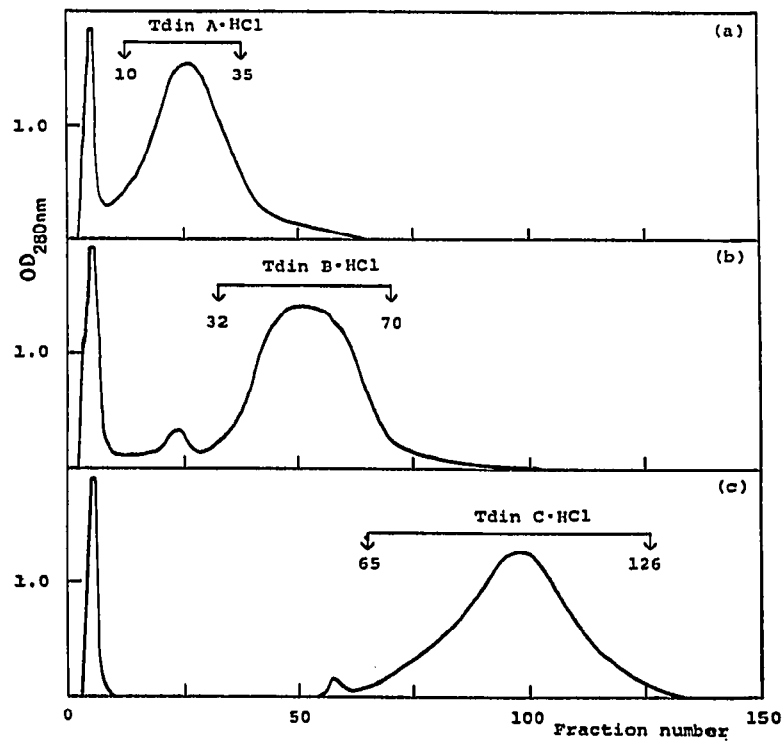


Fig. 7. Re-chromatogram of the partially purified components obtained from PP-Tdin·HCl; (a) PP-Tpin A·HCl; (b) PP-Tdin B·HCl; (c) PP-Tdin C·HCl. Column No., 150; solvent, S_3. One fraction is 4 g.

specific rotations of the peptides were measured (Table IV); in the literature, only data for natural Tdin A·HCl have been reported[6]. The amino acid ratio in each purified peptide agreed closely with the theoretical value (see Table V).

TABLE V

AMINO ACID COMPOSITIONS OF THE PURIFIED TYROCIDINES

Numbers in parentheses give the theoretical amino acid composition.

Amino acid	*Tdin A·HCl*	*Tdin B·HCl*	*Tdin C·HCl*
Asn*	1.00 (1)	1.01 (1)	1.02 (1)
Gln*	0.94 (1)	0.98 (1)	0.99 (1)
Pro	1.02 (1)	1.03 (1)	1.02 (1)
Val	0.94 (1)	0.96 (1)	0.99 (1)
Leu	0.96 (1)	0.98 (1)	1.00 (1)
Tyr	0.95 (1)	0.98 (1)	0.97 (1)
Phe	2.99 (3)	1.89 (2)	1.05 (1)
Trp	0.00 (0)	0.88 (1)	1.73 (2)
Orn	1.00 (1)	1.00 (1)	1.00 (1)

* Asparagine and glutamine are determined as aspartic acid and glutamic acid, respectively.

UV and ORD of purified peptides

The UV absorption spectra and ORD curves of purified Gdin and Tdin are shown in Figs. 8 and 9. In both experiments, tryptophan and Gdin S are included as control. As seen in Fig. 8, tryptophan residues in Gdin A, B and C and Tdin B and C appear not to be oxidized during DCCC and the subsequent isolation.

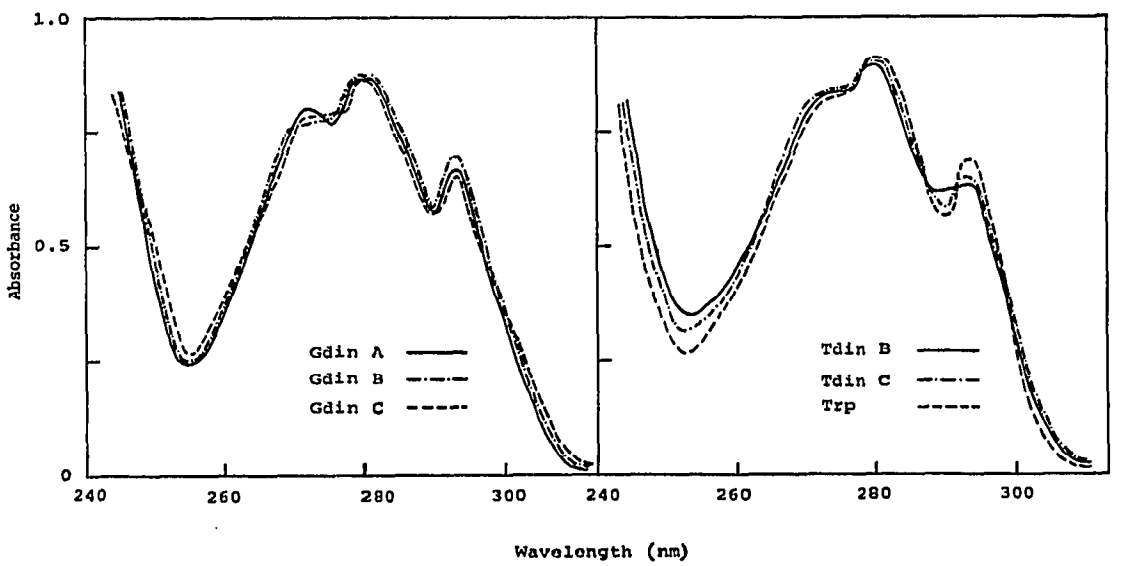


Fig. 8. UV absorption spectra of the purified peptides and tryptophan.

TABLE IV

YIELDS AND SOME PROPERTIES OF THE PURIFIED TYROCIDINES

Compound	*Yield of PP-Tdin·HCl**		*Total yield of PP-Tdin·HCl*		*Yield of Tdin·HCl***		*Total yield of Tdin·HCl*		*M.p. (°C) of Tdin·HCl*	$[\alpha]_D^{25}$ *of Tdin·HCl*
	mg	%	*mg*	%	*mg*	%	*mg*	%		
Tyrocidine A	15	30	48	96	12.3	25	40.3	81	240–242***	−112 °(*c* 0.157, 50% ethanol)§
Tyrocidine B	25	50			21.7	43			238	−106 °(*c* 0.148, methanol)
Tyrocidine C	8	16			6.3	13			246–247	−98 ° (*c* 0.115, methanol)

* PP-Tdin·HCl (50 mg) was subjected to DCCC (see Fig. 6).

** Each of PP-Tdin A·HCl (15 mg), PP-Tdin B·HCl (25 mg), and PP-Tdin C·HCl (8 mg) was re-chromatographed, respectively (see Fig. 7).

*** Reported value, 240–242 ° (ref. 6).

§ Reported value, −111 ° (50% ethanol) (ref. 6).

TABLE VI

ANTIBACTERIAL ACTIVITY OF THE PURIFIED PEPTIDES

The assays were carried out with a bouillon agar medium. Numerals in parentheses give the concentration with a synthetic agar medium.

Microorganism	*Minimum inhibitory concentration (μg/ml)*						
	Gdin B	*Gdin A*	*Gdin C*	*Tdin A·HCl*	*Tdin B·HCl*	*Tdin C·HCl*	*Gdin S·2HCl*
*Escherichia coli**	>50 (>50)	>50 (>50)	>50 (>50)	>50 (>50)	>50 (>50)	>50 (>50)	>50 (>50)
Staphylococcus aureus	5 (5)	20 (50)	1 (2)	10 (10)	20 (50)	2 (2)	2 (2)
Bacillus subtilis	20 (50)	50 (50)	10 (20)	5 (5)	20 (20)	1 (2)	1 (2)

* All peptides showed no activity towards *Proteus vulgaris* and *Mycobacterium Takeo*.

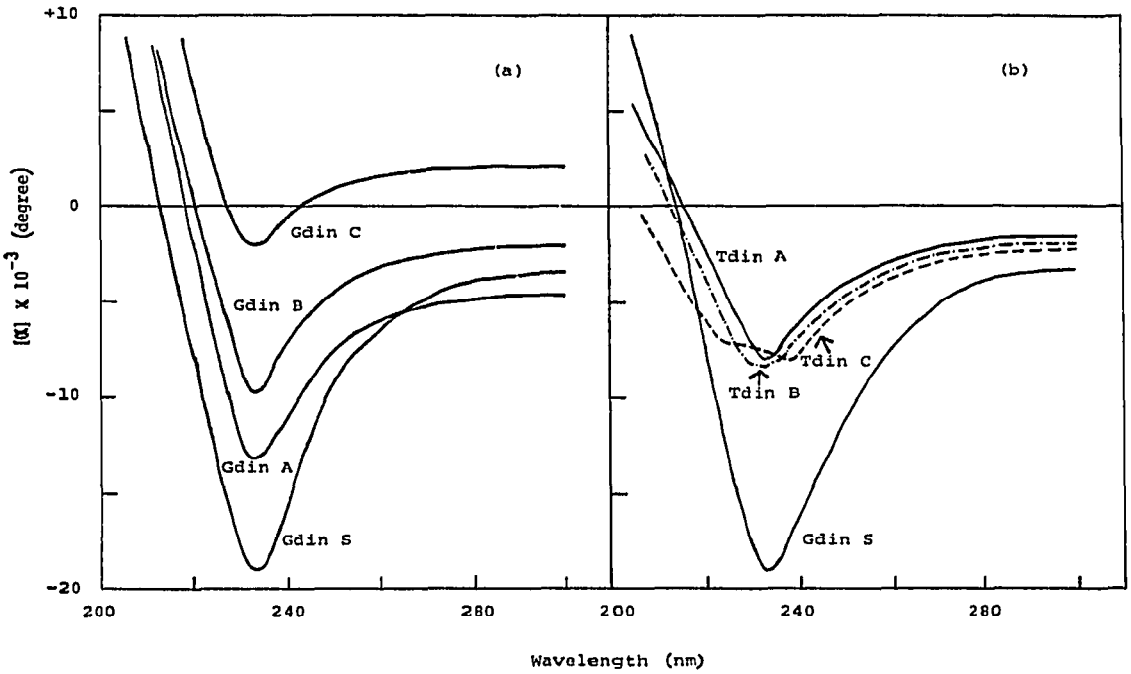


Fig. 9. ORD curves of the purified peptides.

Gdin A, B and C gave similarly shaped ORD curves with a trough at 233 nm, such as Gdin S possesses (Fig. 9a) although the troughs are shallower than that of Gdin S. Tdin A and B also gave a similarly shaped ORD curve as Gdin S, but Tdin C possesses two troughs at 224 and 237 nm; the complex curve of Tdin C may be due not only to the conformation of the main peptide chain but also to an additional effect of two tryptophan residues in a small cyclic peptide. It should be noted that Ruttenberg *et al.* have reported the ORD curve of Tdin B[19].

It is assumed that Gdin S has a β-folded sheet structure, having an antiparallel tripeptide sequence with four hydrogen bond[20–23], but it is difficult to conclude whether Tdin A, B and C have conformations similar to that of Gdin S by the ORD experiment alone.

Antibacterial activity of purified peptides

The inhibition of Gram-positive microorganisms (*e.g.*, *Staphylococcus aureus*) is slightly stronger for the hydrophilic Gdin C than for Gdin A and B (Table VI); Gross and Witkop have already observed a similar trend[2]. Tdin C appears to possess stronger activity than Tdin A and B, but no explanation for this can be made at present.

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