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PURIFICATION OF VANADIUM BINDING SUBSTANCE FROM THE BLOOD CELLS OF THE TUNICATE, ASCIDIA SYDNEIENSIS SAMEA

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Summary. Vanadium binding substance has been partially purified through chromatographies on Sephadex G-25 and SE-Cellulose at pH 2.3. The binding substance was colorless, relatively stable and maintained vanadium ion. The vanadium ion in the substance existed in vanadyl form (VO(IV)). Furthermore, the substance had an apparent affinity for exogenous vanadium ion(V) and contained a reducing sugar.

Although over 70 years have passed since the initial finding of vanadium in the blood cells of the ascidian (1), and although vanadium has been found at concentrations as high as 40mM within these cells (2-6), not only have the physiological roles of the vanadium ion in ascidians not been elucidated but the vanadium binding substance has not yet been isolated from ascidian blood cells. Since tunichromes contained in the ascidian blood cells have the ability to reduce the vanadium ion(V) to its tetravalent oxidation state (7, 8), many efforts have been directed towards the isolation and determination of the chemical structures of the tunichromes (2, 7, 9-12). There have been no reports of the isolation of vanadium binding substance since the studies by Swinehart et al. (3) and Macara et al. (12). However, it has recently been demonstrated that the morula cells, previously thought to be the vanadocytes involved in the accumulation of vanadium ions, contain very few or no vanadium ions, while signet ring cells which contain the lowest concentrations of tunichromes (11) accumulate large amounts of vanadium ions (14-16). Thus, there is a possibility that tunichromes are not involved in the accumulation of vanadium in ascidian blood cells.

We have attempted to extract and purify a vanadium binding substance from ascidian blood cells, and we found that an ESR signal, characteristic of VO(IV)

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derived from the putative binding substance, did not change its pattern as the purification proceeded through several chromatographic stages. The binding substance had an apparent affinity for exogenous vanadium ion(V), and the substance itself appeared to contain a reducing sugar.

Materials and Methods

Ascidia sydneiensis samea were collected from tetrapods, near the Asamushi Marine Biological Station, Tohoku University, Aomori, Japan. Blood, drawn by making an incision through the lower part of the tunic and puncturing the mantle, was separated into blood cells and blood plasma fractions by centrifugation at 5000g for 20min at 4°C. About 2.4g of cell pellet (wet weight) was suspended in 10mM glycine-HCl buffer solution at pH 2.3. The suspension was exposed to an ultrasonicator at 23.5KHz for 5sec, and was then ground in a glass-Teflon homogenizer. The homogenate was loaded onto a column (2.2cm ϕ × 42.0cm) of Sephadex G-25 that was equilibrated in the glycine-HCl buffer. The column was eluted with this same buffer and the vanadium in each 5ml fraction was measured by neutron activation analysis (6). Fractions that contained vanadium were pooled and loaded, for ion-exchange chromatography, onto a column of SE-Cellulose (2.2cm ϕ×17.0cm) for further purification. After non-absorbed substances were washed off the column by the buffer solution, vanadium binding substance was eluted with a linear gradient of KCl (0M to 0.25M) in the buffer solution and the vanadium in each fraction was also measured. A part of each fraction was analyzed for its reducing sugar content by the method of Dubois et al. (17). Determination method of vanadium by neutron activation analysis and measurement of ESR spectra were described previously (6, 16).

Results and Discussion

As shown in Fig. 1, the peak of fractions that contained vanadium, invariably eluted from Sephadex G-25 one fraction prior to the peak of UV absorbance at 256nm. Neutron activation analysis revealed that a total of 11.9μg of vanadium was contained in fractions 21 through 27, a value which correponds to about 67% of the initial amount (17.7μg) in the homogenate loaded onto the column. The peak of vanadium ions coincided with a peak of a reducing sugar, which amount contained in fractions 21 through 30 was 112.0μg, as calculated in term of glucose. The UV spectrum of the peak that contained vanadium is depicted in Fig. 2, shows a maximal absorbance at 256nm. No absorption was detected in the visible portion of the spectrum. On the other hand, both crude and purified tunichromes have been reported to produce absorption peaks at 280, 335 and/or 340nm (2, 3, 12, 18). The spectrum shown in Fig. 2 is clearly different from those of the tunichromes.

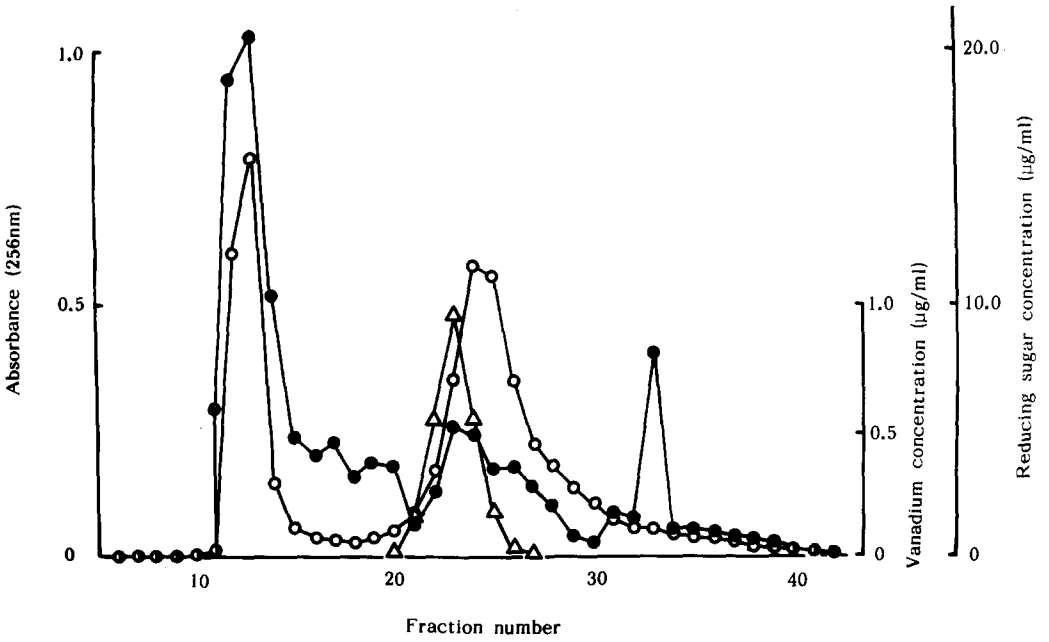

Figure 1. Fractionation of Blood Cell Homogenate from Ascidia sydneiensis samea on Sephadex G-25.
Pelleted blood cells were homogenated in 10mM glycine-HCl buffer solution at pH 2.3. The homogenate was loaded onto a column (2.2cm ϕ × 42.0cm) and eluted with the same buffer solution at 5ml per each fraction. ○:Absorbance at 256nm, △:Vanadium concentration (μg/ml), ●:Reducing sugar concentration (μg/ml)

Fig. 3 shows the results of ion-exchange chromatography on SE-Cellulose. The peak of fractions that contain vanadium, from fractions 83 through 91, coincides with a peak of UV absorbance at 256nm. This peak produced a UV spectrum similar to that the peak that contained vanadium that was separated on Sephadex G-25, as shown in Fig. 2, and it also contained a reducing sugar which was present as an overlapping peak with the peak of vanadium ions. The amount contained this peak was 78.4μg. 4.8μg of vanadium were recovered from the fractions, a value which corresponds to about 27% of that in the initial homogenate.

The intracellular pH values of ascidian blood cells have been reported to diverge widely, and values from less than pH 3.0 to neutral pH have been published (1, 19-22). However, because the pH value of the blood lysate of this species was measured as 2.3, the buffer solution for our experiments was adjusted to pH 2.3 throughout our experiments. In fact, when a buffer solution at neu-

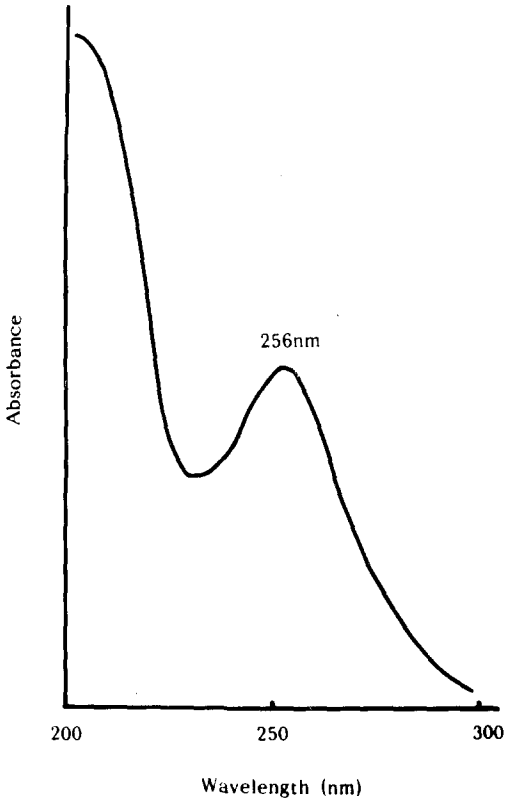

Figure 2. Ultraviolet Spectrum of Vanadium Containing Peak as Eluted from Sephadex G-25.

tral pH was used for the purification of vanadium binding substance, chromatography on Sephadex G-25 gave irreproducible results.

An ESR signal characteristic of VO(IV) can be detected in ascidian blood cells (3, 8, 23, 24). A homogenate of the blood cells of this species shows an ESR signal due to VO(IV) both at liquid nitrogen and room temperatures (Fig. 4a) which resembles that observed in the living vanadocytes of Ascidia ahodori (16). This result suggests that intracellular VO(IV) is not bound to a macromolecule but to a small molecule. Although the intensity of the signal became weaker as the purification proceeded, patterns similar to this signal could be obtained in fractions separated on Sephadex G-25 and on SE-Cellulose (Figs. 4b and 4c). The ESR parameters observed in these fractions were summarized in Table I. After these fractions were allowed to stand for 10 days in an air-exposed condition at 4°C, an ESR signal for VO(IV) could be observed clearly.

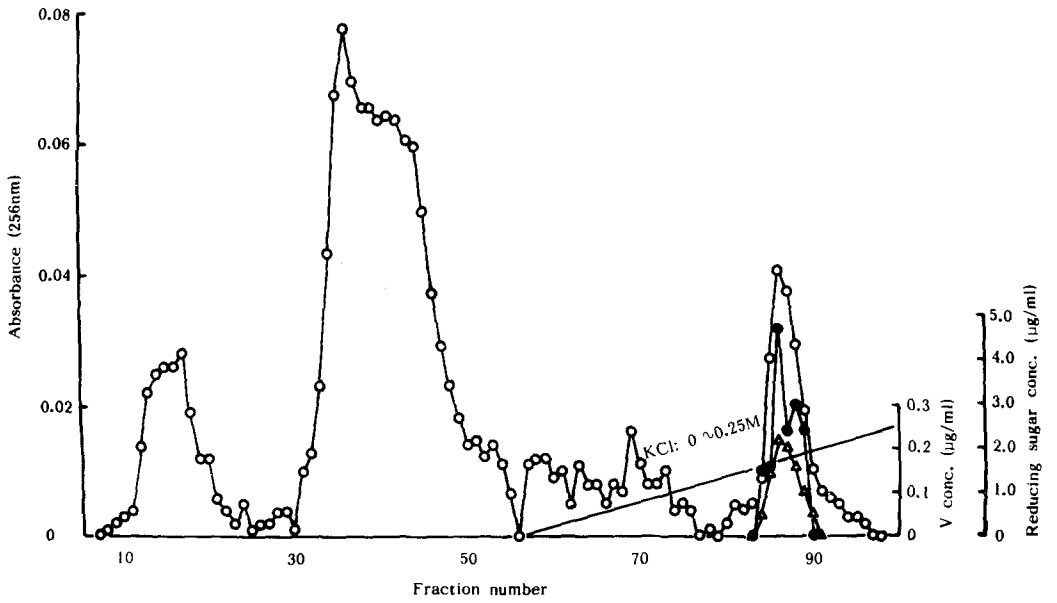

Figure 3. Isolation of Vanadium Binding Substance on SE-Cellulose Column.
Eluted fractions from Sephadex G-25, that contained vanadium ion, were loaded onto a column (2.2cmϕ ×17.0cm) and eluted with a linear gradient of KCl (0M to 0.25M) in glycine-HCl buffer solution at pH 2.3 at 5ml per each fraction. O:Absorbance at 256nm, Δ:Vanadium concentration (μg/ml), ●:Reducing sugar concentration (μg/ml)

These results suggest that the vanadium binding substance is relatively stable, and that it maintains vanadium in a reduced state.

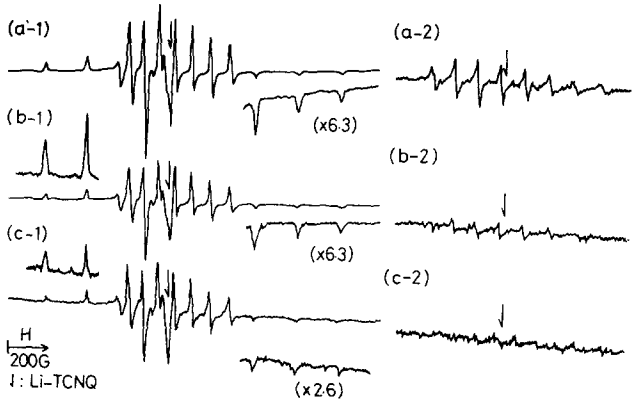

Figure 4. ESR Spectra of Vanadyl Ion.
The spectra were detected in homogenate (a), fractions separated on Sephadex G-25 (b) and on SE-Cellulose (c) of blood cells of Ascidia sydneiensis samea at liquid nitrogen temperature (left side) and room temperature (right side). Instrument conditions: microwave power, 5mW; modulation width, 6.3G; microwave frequency, 9.15GHz; amplitude, 160 for (a-1), 320 for (b-1), 1250 for (c-1), 3200 for (a-2), (b-2) and (c-2).

Table I. ESR Parameters of Vanadyl Ion Detected in Homogenate and Peaks of Vanadium Containing Fractions Separated both on Sephadex G-25 and SE-Cellulose of Blood Cells of A. sydneiensis samea

Sample	g_o	$g_{//}$	$g_\perp$	A_o	A//	$A_\perp$
				Gauss ($\times 10^{-4}cm^{-1}$)		
Blood Homogenate	1.983	2.001	1.974	115 (106)	201 (188)	72 (66)
Fractions separated on Sephadex G-25	1.983	1.943	2.003	116 (108)	200 (181)	75 (70)
Fractions separated on SE-Cellulose after G-25	-	1.944	-	-	201	-

The values of $g_{//}$ and $A_{//}$, and g_o and A_o were obtained from the ESR spectra measured at liquid nitrogen temperature and room temperature (22°C), respectively. The values, $g_\perp$ and $A_\perp$, were calculated by the following equations: $g_o=(g_{//}+2g_\perp)/3$ and $A_o=(A_{//}+2A_\perp)/3$.

Furthermore, the fractions that contained vanadium, after chromatography on Sephadex G-25 and on SE-Cellulose, had apparent affinities for exogenous vanadate ion(V). When 320μg of vanadate ion (Na_3VO_4) were added to a homogenate of blood cells (2.4g of wet weight), 115μg of vanadium ion could be recovered exclusively from the fractions that contained the intrinsic vanadium ion on Sephadex G-25, at a yield of about 36%. Further, 13% of the initial amount of vanadium ion added to the homogenate was recovered from the fractions that contained the intrinsic vanadium ion after elution from SE-Cellulose.

Based on these results, it was concluded that (1) vanadium binding substance in the blood cells of A. sydneiensis samea could be partially purified by combination of Sephadex G-25 and SE-Cellulose columns at pH 2.3, monitoring by vanadium ion by means of neutron activation analysis, (2) the vanadium binding substance was colorless and assumed to have a low-molecular weight, and (3) the ESR spectra of the purified fractions showed that vanadium ion is exclusively present in the vanadyl form (VO(IV)).

Work on the identification of the chemical nature of the vanadium binding substance is in progress.

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