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ARGININE-SPECIFIC ADP-RIBOSYLTRANSFERASE FROM RABBIT SKELETAL MUSCLE SARCOPLASMIC RETICULUM IS SOLUBILIZED AS THE ACTIVE FORM WITH TRYPSIN: Partial Purification and Characterization[1]

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SUMMARY: Arginine-specific ADP-ribosyltransferase from rabbit skeletal muscle sarcoplasmic reticulum was solubilized as the active form with trypsin. The enzyme was partially purified by subsequent chromatography, successively on DE-52, Con A-Sepharose and Sephadex G-75. An approximately 2,000-fold purification was achieved from the 105,000 x g supernatant of trypsin-treated membrane with a recovery of 2.8%. Dithiothreitol, which activates hen liver nuclear ADP-ribosyltransferase, inhibited the enzyme.

Eukaryotic ADP-ribosyltransferase, an enzyme which catalyzes the transfer of mono(ADP-ribose) moiety from NAD to a specific amino acid in some acceptor proteins forming the mono(ADP-ribose)/acceptor conjugates, is present in several tissues and cells, and based on the acceptor amino acids, the ADP-ribosyltransferases were classified into three types: diphthamide-, arginine- and cysteine-specific enzymes (1). Vertebrate skeletal muscle SR contains arginine-specific ADP-ribosyltransferase (2, 3). We reported that rabbit skeletal muscle SR Ca^{2+}-ATPase, existing as a major protein in the membrane, was ADP-ribosylated by the endogenous ADP-ribosyltransferase, that this modification was dramatically enhanced by adding basic amino acid homopolymers such as poly(L-lysine) and poly(L-ornithine) (4), and that the ADP-ribosylation of Ca^{2+}-ATPase suppressed the enzyme activity (5). These results suggest that Ca^{2+} transport in the SR may be regulated through changes in the rate of ADP-ribosylation of Ca^{2+}-ATPase. Therefore, the

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Abbreviations: SR, sarcoplasmic reticulum; TPCK, N-tosyl-phenylalanyl chloromethyl ketone; HPLC, high performance liquid chromatography.

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association of ADP-ribosyltransferase with the membrane had to be given an attention in order to determine the physiological role of the enzyme.

Soman and Graves (3) reported that ADP-ribosyltransferase activity in the SR was poorly extracted in buffers containing 0.5% Triton X-100 and they suggested that the enzyme activity is due to an integral membrane protein.

During studies on ADP-ribosyltransferase in rabbit skeletal muscle SR membrane, we found that trypsin solubilized the transferase from the SR membrane, as an active form. We report here evidence for the trypsin-directed solubilization of ADP-ribosyltransferase from SR membrane and partial purification and characterization of the solubilized enzyme.

MATERIALS AND METHODS

[adenylate-^{32}P]NAD (29.6 TBq/mmol) was obtained from New England Nuclear. NAD, poly(L-arginine) (M.W.24,000), TPCK-treated trypsin from bovine pancreas (Type XIII), and trypsin inhibitor from soybean (Type 1-S) were obtained from Sigma Chemical Co. All other reagents were purchased from Miyata Chemical Co. Ltd, Shimane, and were used without further purification.

Preparation of SR --- The SR from rabbit skeletal muscle was prepared according to the method of MacLennan (6). The final pellet was suspended at a protein concentration of 30 mg/ml in 50 mM imidazole-HCl buffer, pH 7.3.

Solubilization and partial purification of ADP-ribosyltransferase --- The SR at a concentration of 13.6 mg/ml was treated with trypsin at a concentration of 0.27 mg/ml for 30 min at 25°C. The reaction was terminated by adding a trypsin inhibitor at a concentration of 0.14 mg/ml. The preparation was then centrifuged at 105,000 x g for 60 min and the supernatant obtained was placed on DE-52 column (2.6 X 16 cm) pre-equilibrated with 50 mM imidazole-HCl buffer, pH 7.3. After a wash with 800 ml of the equilibrating buffer, the preparation was eluted with 800 ml of the same buffer containing 0.1 M NaCl. The fraction containing enzyme activity was then placed on a Con A-Sepharose column (3.0 X 7.0 cm) pre-equilibrated with 50 mM imidazole-HCl buffer, pH 7.3. After a wash with 500 ml of the equilibrating buffer, the enzyme was eluted with 500 ml of the same buffer containing 0.5 M methyl-α-D-glucoside. The enzyme fraction was then concentrated to 3.0 ml with an ultrafiltration membrane (Amicon, PM 10) and placed on a Sephadex G-75 column (3.0 X 34 cm) pre-equilibrated with 50 mM imidazole-HCl buffer containing 20% glycerol and 1.0 M NaCl. The enzyme was eluted at a rate of 7.0 ml/h and the fraction containing the highest activity of ADP-ribosyltransferase was used for the following experiments.

Analyses --- ADP-ribosyltransferase activity was measured by observing incorporation of the radioactivity from [adenylate-^{32}P]NAD into poly(L-arginine). Unless otherwise stated, the standard assay mixture contained 0.2 mg/ml poly(L-arginine), 50 mM Tris-HCl buffer (pH 8.5), 0.1 mM NAD, [^{32}P]NAD (1.2 MBq/tube), trypsin inhibitor (0.05 mg/ml)[3], and an appropriate amount of enzyme in a total volume of 100 μl. After incubation at 37°C for 30 min, the reaction was terminated by adding excess 10%

[3] As a trace amount of trypsin used for solubilization of ADP-ribosyltransferase from SR membrane was present in the partially purified enzyme preparations, a trypsin inhibitor was added to the assay system. The inhibitor per se did not influence the ADP-ribosyltransferase activity.

trichloroacetic acid and the radioactivity of acid insoluble fraction was counted, as described (7). Protein concentration was measured with Coomassie Brilliant Blue G-250 (8). The molecular weight of the enzyme was estimated by gel filtration, under the conditions used for the enzyme purification, with molecular weight markers, bovine serum albumin (M.W. 67,000), ovalbumin (M.W. 43,000), soybean trypsin inhibitor (M.W. 20,100) and cytochrome c (M.W. 12,384).

RESULTS AND DISCUSSION

Rabbit skeletal muscle SR was treated with varying concentrations of trypsin for 30 min at 25°C. The reaction was terminated with a trypsin inhibitor. An aliquot of the trypsin-treated SR was used directly for ADP-ribosyltransferase assay, another aliquot was centrifuged at 105,000 x g for 60 min and the supernatant was used for the assay of enzyme. Although the total activity decreased over a range of trypsin concentrations, considerable ADP-ribosyltransferase activity was detected in the soluble fraction. As shown in Fig. 1, enzyme activity in the supernatant increased with increasing concentrations of trypsin, and with trypsin concentrations above 30 μg/mg protein of SR the activity progressively decreased. Fig. 2 shows

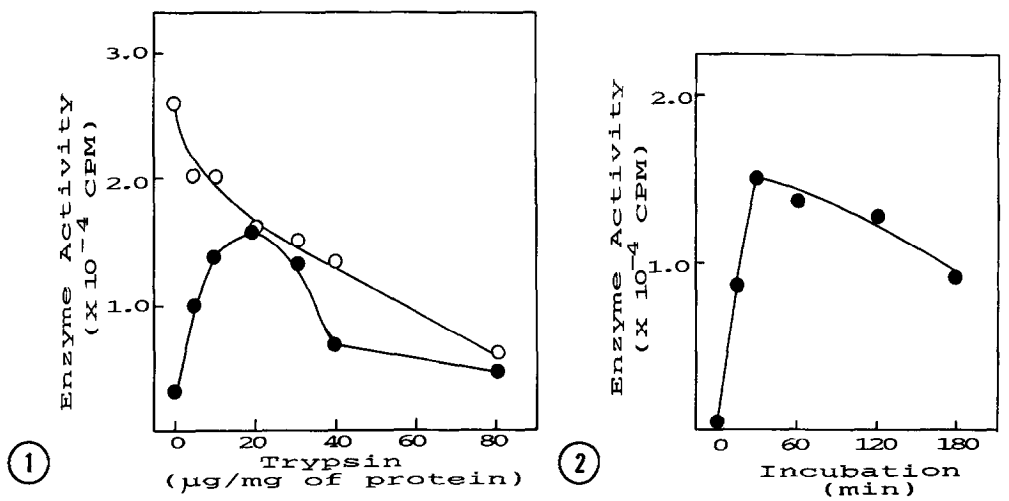


Fig. 1. Solubilization of ADP-ribosyltransferase from SR with varying concentrations of trypsin. Rabbit skeletal muscle SR (13.6 mg/ml) was treated at 25°C for 30 min with varying concentrations of trypsin. Trypsin inhibitor (0.14 mg /ml) was then added into the mixture. An aliquot of the trypsin-treated SR was used for total ADP-ribosyltransferase assay (o), and another aliquot was centrifuged at 105,000 x g for 60 min and the supernatant obtained was used for the determination of the solubilized enzyme activity (●). Other assay conditions were as described under "Methods".

Fig. 2. Solubilization of ADP-ribosyltransferase from SR membrane with trypsin. The SR (13.6 mg/ml) was treated with trypsin (0.27 mg/ml) for the indicated time at 25°C, followed by adding a trypsin inhibitor (0.14 mg/ml). The preparation was then centrifuged at 105,000 x g for 60 min and the supernatant was used for the enzyme assay. Other conditions were as described in the legend to Fig. 1.

the time-dependent solubilization of ADP-ribosyltransferase with 20 μg trypsin/mg protein of SR. Under the conditions described here, the maximal ADP-ribosyltransferase activity was obtained after incubation at 25°C for 30 min.

To determine whether the trypsin-solubilized ADP-ribosyltransferase actually catalyzed the transfer of ADP-ribose from NAD to acceptor to form the ADP-ribose/acceptor conjugate, the solubilized enzyme preparation obtained in the Fig. 2 experiment was incubated with agmatine and [^{32}P]NAD, and the products were analyzed with reverse-phase HPLC, as described (9). We confirmed that the radioactive peak appeared at the time when authentic [^{32}P]ADP-ribosyl agmatine, synthesized with hen liver nuclear ADP-ribosyltransferase (10), was detected. The radioactive product, which we tentatively identified as ADP-ribosyl agmatine, was then treated with 50 mM glycine-NaOH buffer, pH 10.0 for 16 h at room temperature, followed by analysis with HPLC (5), under the same conditions described above. The major radioactive peak appeared at the time obtained with authentic ADP-ribose (data not shown). Thus, the preparation solubilized with trypsin from rabbit skeletal muscle SR membrane apparently catalyzes the arginine-specific ADP-ribosylation reaction.

Next, we attemped to purify the trypsin-solubilized enzyme by chromatography, successively on DE-52, Con A-Sepharose and Sephadex G-75, as described in "Methods". The eluted fraction from Sephadex G-75 gave a single peak of the enzyme activity with an apparent molecular weight of 43,000. This value is 1.6-fold higher than that of ADP-ribosyltransferase

Table 1. Partial purification of ADP-ribosyltransferase from rabbit skeletal muscle SR

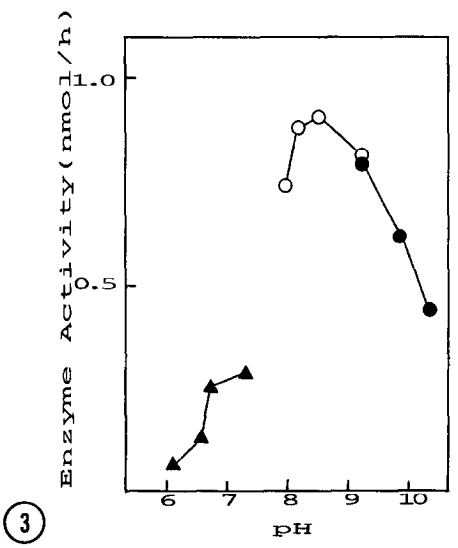
Step	Protein	Total activity	Specific activity	Yield	Purification
	mg	μmol/h	μmol/h/mg	%	-fold
Supernatant	19,754	128.4	0.0065	100	1
DE-52	2,815	76.0	0.027	59.2	4
Con A-Sepharose	86	30.9	0.36	24.1	55
Sephadex G-75	0.28	3.6	12.90	2.8	1,985

Rabbit skeletal muscle SR (23,705 mg) was treated at 25°C for 30 min with 474 mg of trypsin in the total volume 1,738 ml. After preincubation, 250 mg of trypsin inhibitor were added and the mixture was centrifuged at 105,000 x g for 60 min. The supernatant obtained was used for enzyme purification. Other conditions were as described under "Methods".

from hen liver nuclei (10). A summary of a typical purification is shown in Table 1. The gel filtration was most effective for purification of the enzyme, probably due to removal of unidentified inhibitor(s) for the ADP-ribosyltransferase or to separation of the hydrolase (11) catalyzing release of ADP-ribose from the ADP-ribose/acceptor conjugate, from the ADP-ribosyltransferase. We did not investigate this further. An approximately 2,000-fold purification was achieved from the 105,000 x g supernatant of trypsin-treated SR membrane, with a recovery of 2.8%. The partially purified enzyme obtained from gel filtration was used for the following experiments, to characterize the SR ADP-ribosyltransferase.

Effect of pH on the ADP-ribosyltransferase was determined with sodium phosphate, Tris-HCl, and glycine-NaOH buffers, at a concentration of 50 mM. The optimum pH was observed at pH 8.5. As noted with the hen liver nuclear enzyme (10), the SR enzyme also showed that the reaction rate with phosphate buffer was lower than with Tris-HCl buffer (Fig. 3).

In 1988, Soman and Graves reported that sulfhydryl reagents inhibited the ADP-ribosylation of SR proteins (3), in contrast to the dithiothreitol-enhanced ADP-ribosylation by hen liver nuclear enzyme (10). We tested this point with partially purified SR ADP-ribosyltransferase and exogenous acceptor poly(L-arginine) and observed that 5 mM and 10 mM dithiothreitol inhibited the enzyme by about 20 and 25%, respectively.



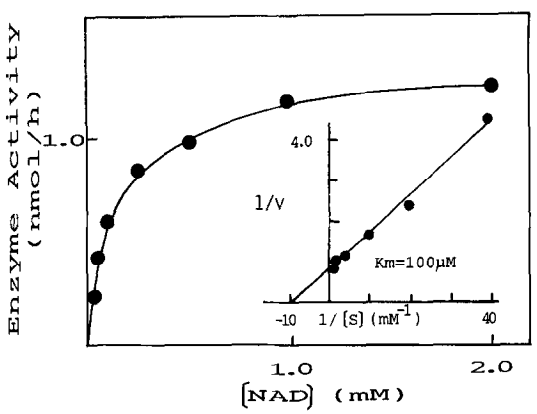


Fig. 3. Effect of pH on ADP-ribosyltransferase. The partially purified enzyme (62.3 ng) was incubated at the indicated pH, as described under "Methods", except that the buffer present in the reaction mixture was 50 mM sodium phosphate (▲), Tris-HCl (○) or glycine-NaOH (●).

Fig. 4. Rate of ADP-ribosylation as a function of NAD concentration. Partially purified enzyme (45.7 ng) was incubated at different concentrations of NAD in the presence of 200 μg/ml poly(L-arginine). Other conditions were as described under "Methods".

The effect of increasing concentrations of NAD with a fixed concentration of poly(L-arginine) on the enzyme activity was determined (Fig. 4). The Km value for NAD was estimated to be 100 μM, that is one fourth that obtained with hen liver nuclear enzyme (10).

Our data support the proposal that arginine-specific ADP-ribosyltransferase from rabbit skeletal muscle SR membrane is solubilized as the active form with trypsin. In 1984, Soman et al. observed the arginine-specific ADP-ribosyltransferase in mammalian skeletal and cardiac muscles (2). We confirmed this, solubilized the enzyme from rabbit skeletal muscle SR with 7% Triton X-100 and partially purified it (12). Comparative studies of the Triton X-100 and trypsin-solubilized enzymes may provide clues as to whether or not SR ADP-ribosyltransferase contains the transmembrane domain.

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