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Purification and Characterization of 2-Oxoglutarate: Ferredoxin Oxidoreductase from a Thermophilic, Obligately Chemolithoautotrophic Bacterium, *Hydrogenobacter thermophilus* TK-6

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2-Oxoglutarate:ferredoxin oxidoreductase from a thermophilic, obligately autotrophic, hydrogen-oxidizing bacterium, *Hydrogenobacter thermophilus* TK-6, was purified to homogeneity by precipitation with ammonium sulfate and by fractionation by DEAE–Sepharose CL-6B, polyacrylate-quaternary amine, hydroxyapatite, and Superdex-200 chromatography. The purified enzyme had a molecular mass of about 105 kDa and comprised two subunits (70 kDa and 35 kDa). The activity of the 2-oxoglutarate:ferredoxin oxidoreductase was detected by the use of 2-oxoglutarate, coenzyme A, and one of several electron acceptors in substrate amounts (ferredoxin isolated from *H. thermophilus*, flavin adenine dinucleotide, flavin mononucleotide, or methyl viologen). NAD, NADP, and ferredoxins from *Chlorella* spp. and *Clostridium pasteurianum* were ineffective. The enzyme was extremely thermostable; the temperature optimum for 2-oxoglutarate oxidation was above 80°C, and the time for a 50% loss of activity at 70°C under anaerobic conditions was 22 h. The optimum pH for a 2-oxoglutarate oxidation reaction was 7.6 to 7.8. The apparent K_m values for 2-oxoglutarate and coenzyme A at 70°C were 1.42 mM and 80 μM, respectively.

Hydrogenobacter thermophilus TK-6 (IAM 12695) is an extremely thermophilic, aerobic, obligately autotrophic, hydrogen-oxidizing bacterium that was isolated in our laboratory (9). Besides having such unique cell components as 2-methylthio-1,4-naphthoquinone (8), the strain can also be characterized by the ability to fix carbon dioxide via an unusual pathway for an aerobic organism, namely, a reductive tricarboxylic acid cycle (17). Among aerobes, the cycle has also been reported to be operative in *Calderobacterium hydrogenophilum* (11) as a carbon dioxide fixation pathway. Among anaerobes, the cycle has been reported to be operative in *Chlorobium limicola* (1), *Desulfobacter hydrogenophilus* (16), and *Thermoproteus neutrophilus* (2).

To confirm the operation of the reductive tricarboxylic acid cycle and to characterize the cycle in *H. thermophilus*, it is very important to purify the key enzymes involved in this cycle. 2-Oxoglutarate:ferredoxin oxidoreductase (OGOR [EC 1.2.7.3]), pyruvate:ferredoxin oxidoreductase (POR, [EC 1.2.7.1]), and ATP:citrate lyase are the key enzymes of the reductive tricarboxylic acid cycle. ATP:citrate lyase from this strain had already been purified and characterized (7), but purification of OGOR or POR from the strain has not been demonstrated. OGORs were only purified from a photosynthetic bacterium, *Chlorobium thiosulfatophilum* (6), and an archaebacterium, *Halobacterium halobium* (10).

This report describes the purification and characterization of OGOR from *H. thermophilus* TK-6. This report is the first report on the purification of OGOR from an aerobic and obligately chemolithoautotrophic bacterium.

Bacterium, growth conditions, and preparation of cell extract. The *H. thermophilus* type strain, TK-6 (IAM 12695), was grown in a 10-liter fermentor with CO_2 being used as a carbon source and H_2 being used as an energy source according to the method described previously (9). After being harvested, the cells were washed twice with 50 mM phosphate buffer (pH 7.0), and a cell suspension was made by homogenization in the same buffer (1 g [wet weight] of cells per 4 ml of buffer). The cell suspension could be stored frozen at −80°C for up to 1 year before use. The cell suspension was treated with a sonicator (140 W for 5 min) at 4°C. After sonication, cell debris was removed by ultracentrifugation (100,000 × *g* for 1 h). The supernatant fraction was designated cell extract. The cell extract was immediately used for purification or kept at −80°C.

Purification of OGOR from *H. thermophilus* TK-6. OGOR activity was routinely determined spectrophotometrically by anaerobically following the 2-oxoglutarate-dependent reduction of methyl viologen with a cuvette (3.0-ml capacity with a 1-cm light path) at 70°C (18). The cuvette was sealed with a rubber stopper and an aluminum cap, and it was made anaerobic by flushing it with N_2 gas for 3 min. The standard assay mixture (1.5 ml) contained 10 mM sodium 2-oxoglutarate, 0.25 mM coenzyme A, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 5 mM methyl viologen in 100 mM phosphate buffer (pH 7.6). The change in A_{578} (ε_{578} = 9.8 $mM^{-1} \cdot cm^{-1}$) was measured with a U-3200 spectrophotometer (Hitachi, Tokyo, Japan) equipped with a thermostatted cuvette holder and a thermoinsulated cell compartment. The enzyme activity was expressed as units per milligram of protein, with 1 U being defined as 1 μmol of methyl viologen reduced per min. In the assay of POR activity, sodium pyruvate instead of sodium 2-oxoglutarate was used as a substrate. All procedures were carried out at room temperature under aerobic conditions. All the buffers used throughout the purification contained 0.1% Triton X-100, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM sodium dithionite at the indicated pHs. OGOR was routinely purified from 100 ml of cell extract. Cell extract was treated with 1% streptomycin sulfate, and then OGOR was precipitated with ammonium sulfate (35 to 50%) (7). Precipitated proteins were suspended

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in a minimum volume of 10 mM Tris-HCl buffer (pH 8.0) and were treated with a PD-10 column (Pharmacia, Uppsala, Sweden) by using the same buffer as an eluant. The solution (20 ml) was loaded onto a DEAE–Sepharose CL-6B column (4.0 by 20 cm; Pharmacia) preequilibrated with 10 mM Tris-HCl buffer (pH 8.0). The column was washed with 150 ml of the same buffer at a flow rate of 120 ml/h, and then adsorbed proteins were eluted with a linear gradient of NaCl (0 to 1.0 M) in the same buffer. The gradient volume was 500 ml. OGOR activity was eluted at 0.58 to 0.63 M NaCl, and then the active fraction (40 ml) was concentrated with a Centricell-20 apparatus (Polysciences, Inc.). The concentrated enzyme solution was applied to a column (2.0 by 10 cm) of polyacrylate-quaternary amine (Shim-Pack, Tokyo, Japan) preequilibrated with 14 mM Tris-HCl buffer (pH 8.0). The OGOR activity came out at a NaCl concentration of 0.19 to 0.21 M after being eluted at a linear gradient of 0 to 0.4 M NaCl at 5 ml/min. The total gradient volume was 600 ml. The active fraction (35 ml) was concentrated, and then the buffer was exchanged to 1 mM potassium phosphate buffer (pH 6.8) with a Centricell-20. The concentrated enzyme solution was loaded onto a hydroxyapatite chromatography column (0.5 by 10 cm; Mitsui Toatsu Corporation, Tokyo, Japan) preequilibrated with 1 mM potassium phosphate buffer (pH 6.8). The OGOR activity was eluted at 105 to 120 mM potassium phosphate after sequential gradients of 1 to 100 mM (20 ml), 100 to 150 mM (20 ml), and 150 to 400 mM (20 ml) potassium phosphate were used. The active fraction (6 ml) was concentrated to approximately 1.5 ml with Ultra-Free-CL (Millipore). The concentrated protein was loaded onto a Superdex-200 column (1.0 by 60 cm; Pharmacia) preequilibrated with 150 mM potassium phosphate buffer (pH 7.2) containing 200 mM NaCl, and the protein was eluted with the same buffer at 1.0 ml/min. The active fractions were stored under N_2 gas at −20°C. All the subsequent assays were performed without the Triton X-100 being removed. The protein concentration was routinely estimated by the Lowry method, with bovine serum albumin being used as a standard protein (14).

OGOR activity could not be detected in the particulate fraction by ultracentrifugation, which indicates that the enzyme from *H. thermophilus* is a cytoplasmic one. Both OGOR and POR activities were recovered at between 35 and 50% saturation by ammonium sulfate fractionation. However, by DEAE–Sepharose CL-6B chromatography, OGOR activity was separated from POR activity (Fig. 1). In polyacrylate-quaternary amine anion-exchange chromatography, the fractions of OGOR activity could be completely distinguished from those of POR activity (20). This fact clearly shows that *H. thermophilus* apparently possesses two distinct enzymes responsible for OGOR and POR activities. OGOR was purified 75-fold from cell extract of *H. thermophilus*, with a 16% recovery (Table 1).

Polyacrylamide gel electrophoresis (PAGE) was carried out with a 4 to 20% (wt/vol) gradient gel in the absence of sodium dodecyl sulfate (SDS) (5). SDS-PAGE was carried out with a 12.5% gel (12). Activity stains of nondenaturating gels were carried out anaerobically under 10 mM 2-oxoglutarate, 0.25 mM coenzyme A, 1 mM $MgCl_2$, and 5 mM triphenyltetrazolium (which was used as an electron acceptor) in 100 mM phosphate buffer (pH 7.2) at 70°C. The purified enzyme gave a single protein band after electrophoresis on a nondenaturating gel and total protein staining (Fig. 2, lane a). The activity stain showed that the purified enzyme was responsible for the activity (Fig. 2, lane b). SDS-PAGE showed that OGOR comprised two subunits with molecular masses of 70 and 35 kDa, indicating an αβ structure (Fig. 2, lane c). The molecular mass of the

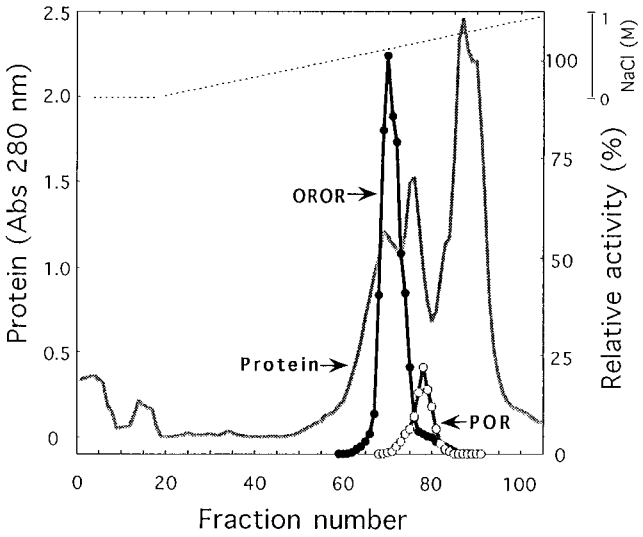

FIG. 1. Elution patterns from DEAE–Sepharose CL-6B chromatography during purification of OGOR from *H. thermophilus*. The elution rate was 120 ml/h, and the fraction volume was 6.0 ml. The broken line indicates a linear gradient of 0 to 1.0 M NaCl. Abs, absorbance.

purified OGOR from *H. thermophilus* TK-6 was estimated to be approximately 105 kDa by Superdex-200 and Superose 12 gel filtration chromatography. The molecular masses of OGOR from *C. thiosulfatophilum* (6) and *H. halobium* (10) were 220 and 230 kDa, respectively. In the case of *H. halobium*, its OGOR was a tetramer comprising two 88-kDa and two 36-kDa subunits. OGOR from *H. thermophilus* is clearly different from that of *H. halobium* in terms of molecular weight and subunit structure.

The N-terminal amino acid sequences of the subunits of OGOR were determined with a gas-phase ABI Protein/Peptide Sequencer (Applied Biosystems model 477A) equipped with a phenylthiohydantoin analyzer (Applied Biosystems model 120A) for the on-line detection of phenylthiohydantoin amino acids (15). The N-terminal amino acid sequences of each subunit of OGOR were as follows: α subunit, AFDLTIK IGGCGGWGVIEAGDFLTI; and β subunit, MLEVHLKPA DTKSDVEPTWS. We could not find any similar proteins.

General properties of OGOR. The purified OGOR had an absorption maximum at around 405 nm, which is a general characteristic of ferredoxin-dependent enzymes possessing Fe-S clusters (10). OGOR was sensitive to inactivation by oxygen, which made it difficult to purify the enzyme under aerobic conditions. However, we could purify the enzyme in the air at room temperature, because we found that the enzyme could be highly stabilized by the addition of 0.1% Triton X-100 to the purification buffers. The addition of the detergent

TABLE 1. Purification of OGOR from *H. thermophilus*

Step	Protein (mg)	Activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Cell extract	598	145	0.23	100	1
Ammonium sulfate	404	126	0.31	87	1.3
DEAE–Sepharose CL-6B	98	76	0.78	52	3.2
Polyacrylate-quaternary amine	8.6	47	5.5	32	23
Hydroxyapatite	1.7	28	16	19	67
Superdex-200	1.3	23	18	16	75

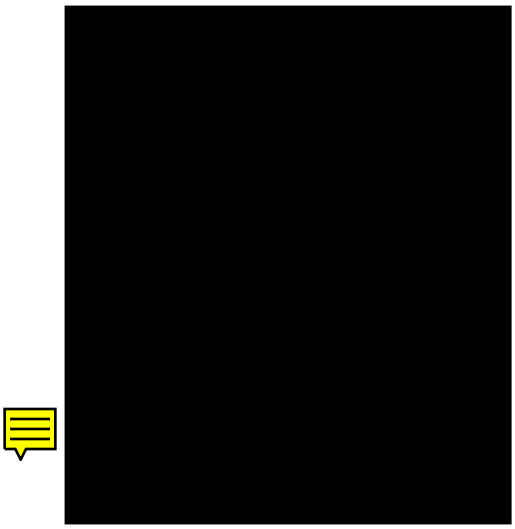

FIG. 2. Native 4-to-20%-gradient PAGE and SDS–12.5% PAGE of the purified OGOR from *H. thermophilus*. Lanes: a, protein stain of 2.0 μg of purified protein; b, activity stain of 2.0 μg of purified protein; c, 3.0 μg of denaturated OGOR; d, low-molecular-weight standard proteins (M_rs, 14,400 to 94,000).

gave no effect on the enzyme activity directly. We do not know the precise mechanism of the stabilization of OGOR by Triton X-100. At present, we presume that the redox active center (Fe-S cluster) within the disulfide bridge by the cysteine was protected against attack by oxygen. The optimum pH for OGOR activity was in the range of from 7.6 to 7.8, and the enzyme was more stable in alkaline rather than acidic pH ranges. The purified OGOR showed a dramatic increase in activity above 60°C, with the optimum being at around 80 to 85°C. The time for a 50% loss of activity at 70°C was 22 h under anaerobic conditions. Under aerobic conditions, OGOR showed only a 50% residual activity after treatment at 60°C for 30 min. The K_m value for each reaction component was determined by the double-reciprocal plot method (13) under optimum pH and with the condition that the other components exist in optimum concentrations. The apparent K_m values at 70°C and pH 7.6 for 2-oxoglutarate and coenzyme A were determined to be 1.42 mM and 80 μM, respectively.

Specificities for substrates and electron acceptors of OGOR. The purified OGOR did not react with oxalacetate, oxomalonate, 2-oxoisocaproate, or phosphoenolpyruvate. The enzyme reacted with 2-oxobutyrate, pyruvate, and 2-oxoisovalerate at an activity level of less than 0.4 to 0.7% relative to that with 2-oxoglutarate (Table 2). The purified POR did not react with 2-oxoglutarate (19). The purified OGOR reacted with methyl viologen, flavin mononucleotide, flavin adenine dinucleotide, and ferredoxin isolated from *H. thermophilus* (19) as electron acceptors. NAD, NADP, and ferredoxin from *Chlorella* spp. (Chlorella Corporation, Tokyo, Japan) or *Clostridium pasteurianum* (Sigma, St. Louis, Mo.) were not reactive (Table 3). The reason why ferredoxins from other sources could not function for the reaction is uncertain, but we presume that the low thermostabilities of those proteins or the differences in the reactivities of ferredoxins may be the reason. The purified enzyme showed no activity for NAD(P)-dependent 2-oxoglutarate dehydrogenase. The fact that OGOR reacted in vitro with ferredoxin isolated from *H. thermophilus* (19) strongly suggested that the ferredoxin is an electron donor for the enzyme reaction in vivo.

TABLE 2. Substrate specificity of OGOR from *H. thermophilus*

Substrate[a]	Relative activity (%)
2-Oxoglutarate	100
2-Oxobutyrate	<0.7
2-Oxoisocaproate	0
2-Oxoisovalerate	<0.4
Oxalacetate	0
Oxomalonate	0
Pyruvate	<0.4
Phosphoenolpyruvate	0

[a] All substrates were used at 10 mM.

TABLE 3. Electron acceptor specificity of OGOR from *H. thermophilus*

Electron acceptor	Concn	Absorbance (nm)	Sp act (U/mg)[a]
Methyl viologen	5 mM	578	18
Flavin mononucleotide	0.25 mM	450	6.3
Flavin adenine dinucleotide	0.25 mM	450	6.9
NAD	5 mM	340	0
NADP	5 mM	340	0
Chlorella ferredoxin	0.35 mg/ml	390	0
C. pasteurianum ferredoxin	0.20 mg/ml	390	0
H. thermophilus ferredoxin	0.15 mg/ml	390	4.5

[a] Activities were calculated with the following absorbance coefficients NAD and NADP, $\varepsilon = 6.2\ mM^{-1}\ cm^1$; flavin adenine dinucleotide and flavin mononucleotide, $\varepsilon = 12.3\ mM^{-1}\ cm^{-1}$; and ferredoxin, $\varepsilon = 17.3\ mM^{-1}\ cm^{-1}$.

In common eubacterial cells, the catalyses of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase are accomplished by similar mechanisms. In archaebacteria and some kinds of thermophilic bacteria, ferredoxin-dependent POR is rather commonly used to catalyze glycolysis (3, 4). On the other hand, OGOR seems to be specific for the reductive tricarboxylic acid cycle. In *H. thermophilus*, POR and OGOR reactions are catalyzed by different enzymes. This fact is highly interesting in a consideration of the evolution of microbial metabolic pathways.

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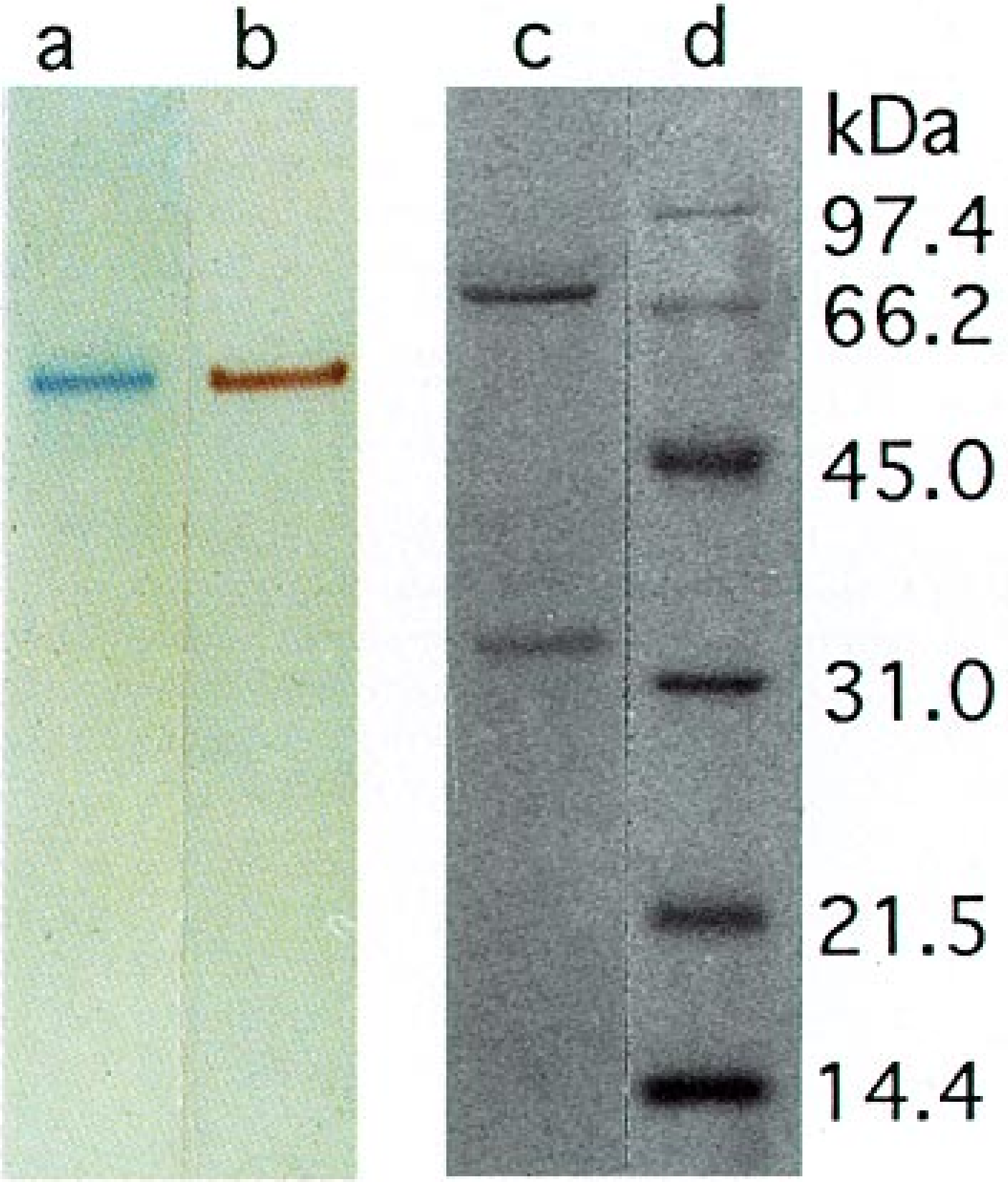
a
b
c
d
kDa
97.4
66.2
45.0
31.0
21.5
14.4