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SOLUBILIZATION OF GLYCOSOMAL OROTATE PHOSPHORIBOSYLTRANSFERASE AND OROTIDYLATE DECARBOXYLASE FROM *CRITHIDIA LUCILIAE*

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Abstract

Tightly bound glycosomal OPRTase and ODCase from Crithidia luciliae *were solubilized using Triton X-100 in the presence of cryoprotectants, dimethyl sulfoxide and glycerol. Recoveries of better than 20% for both enzymes were achieved. Properties of the solubilized enzymes which differed from those of intact glycosomal enzymes included efficiency of substrate channelling, the K_m of ODCase for OMP, heat stabilities and the activation energies obtained from Arrhenius plots. Chromatography on Sepharose CL – 6B in the presence of 0.25 M sucrose yielded a high molecular weight species containing both enzyme activities. In the presence of 0.15 M NaCl and the absence of sucrose, most of the ODCase activity eluted at a position corresponding to a molecular weight of 65,000 with minor peaks of activity associated with higher molecular weight species. OPRTase activity was not recovered.*

Introduction

An unusual feature of the protozoa belonging to the family Kinetoplastida is the occurrence of a unique microbody, the glycosome, which contains the glycolytic enzymes required to convert glucose to α -glycerophosphate and 3-phosphoglycerate. Originally

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observed by Opperdoes and Borst in *Trypanosome brucei*[1], the presence of glycosomes has been described in other members of this order, including *Trypanosoma cruzi*[2], *Leishmania mexicana*[3] and *Crithidia* species[2,4].

The glycosome has subsequently been demonstrated to contain enzymes of other metabolic pathways apart from glycolysis. Notably, Gero and Coombs for *Leishmania mexicana* and *Crithidia fasciculata*[5] and Hammond and Gutteridge[6] for *Trypanosoma cruzi,* reported on the particulate nature of two subsequent enzymes of the *de novo* pyrimidine biosynthetic pathway, orotate phosphoribosyltransferase (OPRTase; EC 2.4.2.10) and orotidylate decarboxylase (ODCase; EC 4.1.1.23). The novel location for these enzymes, which are cytoplasmic in all other known species[7], has been confirmed for other members of this order and shown to be associated with the glycosome[8-10].

We recently reported on the nature of the glycosomal OPRTase-ODcase system in *Crithidia luciliae* and described the high degree of substrate channelling exhibited between these two enzymes[9]. Subsequently, attempts to solubilise and purify OPRTase and ODCase from their membrane bound environment in *C.luciliae* have been made in an attempt to compare their properties with the enzymes from other systems. These experiments are described herein.

Materials and Methods

[Carboxy-^{14}C] orotic acid (32.47 mCi/mmoles) was obtained from New England Nuclear, Massachusetts, U.S.A. [Carboxy-^{14}C] orotidine monophosphate (OMP : 30 mCi/mmole) was synthesized from [Carboxy-^{14}C] orotic acid in the reaction catalysed by yeast OPRTase[10]. N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid (Hepes) was from Calbiochem. Hydroxide of hyamine was supplied by Packard Instrument Co. (Australia). PEI-Cellulose plates were obtained from Macherey-Nagel, Germany. Proteins used as molecular weight markers, namely human spleen ferritin (MW 440,000), rabbit muscle lactate dehydrogenase (EC 1.1.1.28; MW 136,000) and bovine haemoglobin (MW 65,000), were obtained from Sigma Chemical Co., which also supplied phosphoribosyl pyrophosphate (P-Rib-PP). dithiothreitol (DTT), 3- [(3-cholamidopropyl) dimethyl-ammonia] - 1-propane sulphate (CHAPS), deoxycholate, potassium thiocyanate and potassium perchlorate. Triton X-100 (polyoxyethylene octyl phenol with an average of 9.6 ethylene units) was a product of Rohm and Haas, Philadelphia. Other reagents were of the highest commercial quality available.

The cultivation of *C. luciliae* and the preparation of cell free extracts was as described previously[9].

Procedures for the assay of OPRTase, ODCase and hexokinase, and for the substrate channelling experiments, were carried out as described by Pragobpol *et al*[9].

Solubilization of glycosomal enzymes

Fraction M was obtained from differential centrifugation[9]. The postnuclear fraction (1000 g supernatant) was spun at 14,500 g for 15 min at 4°C. After removal of the supernatant, the pellet was washed twice with 5 ml of the buffer used for enzyme extraction and was used as the source of material. Originally, extraction was attempted by suspension in 0.25 M sucrose containing 20mM potassium phosphate, pH 7.4, 0.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 0.5 mM EDTA, to give a final protein concentration of 3-5 mg/ml in a total volume of 3.0 ml. Triton X-100 was added to give final concentration of 0.2% in the suspension. In later experiments, the mixture also contained 30% (v/v) dimethylsulphoxide (DMSO), 5% (w/v) glycerol, 0.2 mM P-Rib-PP and the antifoaming agent, octanol (5 µl). Following freeze-thawing (ethanol-dry ice) three times, the mixture was allowed to stand at 4°C for 45 min and then centrifuged at 139,000 g for 1 h. The supernatant was used as a source of enzymes.

With CHAPS as a solubilizing agent, Fraction M was suspended in 0.25 M surcrose in the presence of 1 mM EDTA, 2 mM DTT and 10 mM potassium phosphate, pH 7.4[12]. Homogenization was carried out at 4°C, in the presence of 5 mM CHAPS, with 25 strokes of a glass homogenizer. Following centrifugation at 139,000 g for 1 h, the supernatant was recovered and assayed for enzyme activity.

Extraction of OPRTase and ODCase was also attempted with deoxycholate, potassium thiocyanate and potassium perchlorate at various concentrations.

The glycosomal hexokinase was solubilized by incubation with Triton X-100 and ATP[1,2,11]. Fraction M suspended in 0.25 M sucrose, 1 mM EDTA, 1 mM DTT, 10 mM potassium phosphate, pH 7.4 to a concentration of 1.7 mg/ml in 2.0 ml, was preincubated with 0.5% (v/v) Triton X-100 at 4°C for 30 min or with 40 mM ATP at 4°C for 50 min. At the end of the incubation period, centrifugation was carried out as above.

Substrate channelling assay for solubilized OPRTase-ODCase

The ingredients for the assessment of the channelling assay by solubilized OPRTase-ODCase were the same as for the glycosomal OPRTase-ODCase (Ref 9), except that the enzyme source was the supernatant obtained by the procedure described in "Solubilization of glycosomal enzymes". The total protein in the reaction mixture was 120 µg.

Stability of Solubilized OPRTase and ODCase on storage

The activities of solubilized OPRTase and ODCase were determined at the time intervals 1, 4, 6, 15 and 30 days after storage at-70°C. The solubilized enzymes had initial specific activities for OPRTase and ODCase of 117 and 80 nmole/mg protein/h, respectively.

Effect of temperature on solubilized OPRTase-ODCase

An Arrhenius plot of the solubilized OPRTase-ODCase activity was determined over the temperature range of 5-38°C. The temperature of the shaking water bath was set at the required temperature (± 0.2°C) and the assay tubes containing the components for the OPRTase-ODCase assay were preincubated at that temperature for 10 min before starting the reaction. Enzyme (115 µg) was added to start the reaction which was allowed to proceed for 15 min. Similar experiments were carried out on the membrane-bound form of the enzymes.

Column chromatography

The solubilized enzymes (3 ml, containing 4.2 mg protein) with specific activities of OPRTase and ODCase of 55 and 59 nmoles/mg protein/h, respectively, were chromatographed on a Sepharose CL-6B column (1.5 × 40 cm). The column had been previously equilibrated at 4°C with 0.25 M sucrose in the presence of 20 mM potassium phosphate, pH 7.4, 1 mM DTT, 0.5 mM EDTA, 30% (v/v) DMSO and 5% (w/v) glycerol (Buffer A). The same buffer was used to elute the enzymes and 3.0 ml fractions were collected.

Chromatography was also carried with Buffer B, which contained the same components as Buffer A except that 0.15 M NaCl and 0.5 mM $MgCl_2$ were added and sucrose omitted. For this experiment, 3.0 ml containing 5.4 mg protein, with specific activities of 63 and 65 nmoles/mg protein/h for OPRTase and ODCase, respectively, was loaded onto the column.

Protein

Protein estimations were carried out by the Coomassie Blue procedure of Bradford[13].

Results

Solubilization of enzymes

In the absence of cryoprotectants, attempts to solubilize OPRTase and ODCase activities from the *C. luciliae* glycosomes, with various detergents and chaotropic agents gave very poor yields, of the order of 0.1 to 0.5%. Further, Triton X-100, which has been reported to disrupt the glycosomal membrane, with subsequent expression of latent activity for most enzymes of glycolysis[1,2,11], was inhibitory to both OPRTase and ODCase activities (cf. Ref. 11). The effect was greater on the former enzyme, increased with increasing Triton concentration and with decreasing protein concentration. On the

other hand, approximately 20% recovery of hexokinase was obtained following treatment with 0.5% Triton for 30 min at 4°C. The yield was increased to 50% by preincubation of the glycosomes with 40 mM ATP for 90 min at 4°C.

The recoveries of OPRTase and ODCase with Triton (0.2%) were, however, considerably improved in the presence of glycerol and DMSO. Optimal concentrations of these two cryoprotectants were determined as 5% and 30%, respectively (Fig. 1).

Of the other reagents tested, only CHAPS gave reasonable recoveries, 5 and 17 nmoles/mg protein/h for OPRTase and ODCase, respectively. The experiments described below were carried out using enzyme obtained from the Triton treatment. The percentage recoveries from this treatment were consistently in the range of 20-30% (Table 1).

Properties of solubilized enzymes

Specific activities of 69-117 and 80-127 nmoles/mg protein/h were observed for the solubilized OPRTase and ODCase, respectively. Both enzymes were stable for at least four weeks when stored at-70°C. However, OPRTase was very heat labile, activity being lost completely at 55°C for 5 min at a protein concentration of 0.8 mg/ml. P-Rib-PP (2.0 mM) and DTT (1.5 mM) failed to protect the activity. ODCase was only slightly less heat labile, 10% of activity being retained after 5 min at 55°C. 6-AzaUMP conferred a slight degree of protection against inactivation.

Kinetic parameters for the solubilized enzymes varied only slightly from those obtained with intact glycosomes[9]. The degree of substrate (OMP) channelling was, however, significantly reduced. This behaviour is illustrated in Fig. 2, which should be compared to Fig. 4 of Ref. 9. A calculation of the comparative degree of preference by ODCase for endogenous OMP, with respect to exogenous OMP, gave a value of 4 for the channelling factor, compared to 50 for the enzymes bound to the intact glycosomes (see Table III of Ref. 9).

An Arrhenius plot for the solubilized OPRTase-ODCase system demonstrated a discontinuity at approximately 18°C, with activation energies of 9.6 and 9.8 kcals below and above the transition point, respectively. The discontinuity was much less dramatic than that observed with the membrane bound enzymes, which gave a transition at 15°C, with activation energies of 12.4 and 6.8 kcal, respectively, below and above the transition point.

Column chromatography

The behaviour of the solubilized enzymes on Sepharose CL-6B varied according to the nature of the eluting buffer. In the presence of dilute (20 mM) phosphate buffer,

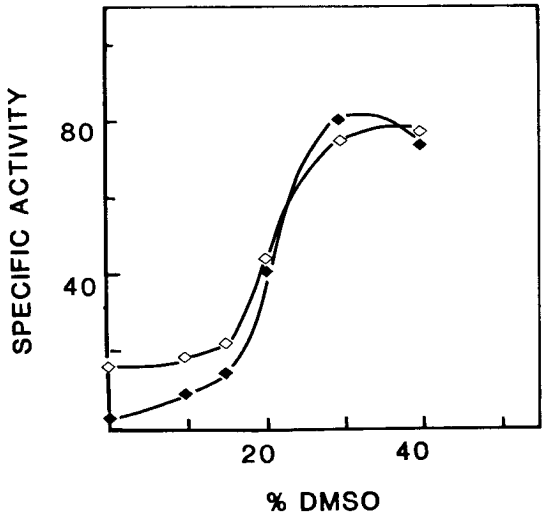

Figure 1. Effect of DMSO on the activity (recovery) of OPRTase (■——■) and ODCase (□——□) extracted from Fraction M by 0.2% Triton X-100 in the presence of 5% glycerol. The extraction medium also contained 0.25 M sucrose, 1 mM EDTA, 2 mM DTT, 10 mM potassium phosphate, pH 7.4, 5 µl octanol and 4.7 mg protein in a total volume of 1.0 ml. Activities are expressed as nmoles/mg protein/h.

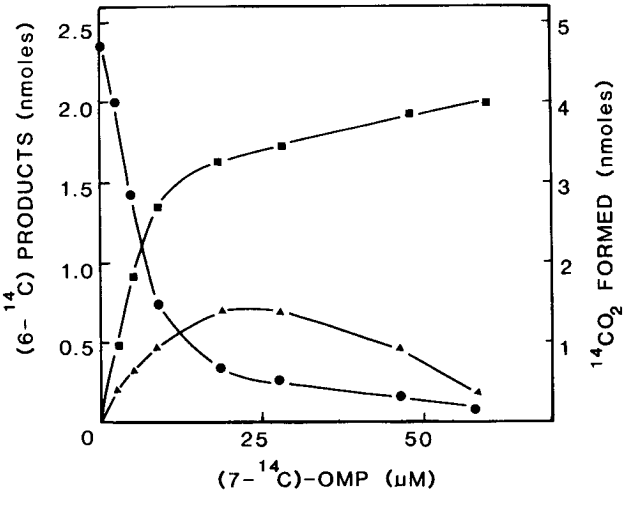

Figure 2. Substrate channelling of solubilized OPRTase-ODCase. The utilization of exogenous [7-^{14}C] OMP by ODCase was determined by the release of $^{14}CO_2$ (■——■). The synthesis of [6-^{14}C] UMP (●——●) represents the overall activity of the OPRTase-ODCase system in converting orotate to UMP via OMP. [6-^{14}C] OMP (▲——▲) measured the internal OMP formed in the presence of exogenous [7-^{14}C] OMP during the incubation time of 15 min. (See Fig. 4 of Ref. 9).

Table 1. Recovery of glycosomal OPRTase and ODCase extracted with Triton X-100 in the absence and presence of cryoprotectants (30% v/v DMSO and 5% w/v glycerol)

Sample	Protein (mg/ml)	Specific activity (nmoles/mg protein/h)		Total activity (nmoles/h/ml)		Percentage recovery	
		OPRTase	ODCase	OPRTase	ODCase	OPRTase	ODCase
Fraction M	3.0	103	161	309	483	100	100
Extraction without Cryoprotectants							
Pellet	1.7	20	53	34	90	11	18
Supernatant	0.8	12	10	10	8	3	2
Extraction with Cryoprotectants							
Pellet	2.1	32	52	64	111	21	23
Supernatant	0.9	69	127	62	114	20	24

cryoprotectants and 0.25 M sucrose, both enzymes eluted at a position just after the void volume, corresponding to a molecular weight of $\geq$ 500,000 (Fig. 3). Activities of both enzymes in this high molecular weight form were moderately stable, losing 50% of their activity after storage at -70°C for one week.

Omission of the sucrose and the addition of high salt (0.15 M NaCl) gave a very different profile. OPRTase activity was completely lost and most of the ODCase activity eluted at a position corresponding to a molecular weight of approximately 65,000, with smaller peaks at higher molecular weights (Fig. 4). Reduction of the salt concentration to 0.05 M substantially reduced the amount of enzyme in the lowest molecular weight form, with corresponding increases in the higher molecular weight forms.

Properties of the low molecular weight form of ODCase

The ODCase activity in the peak C (Fig. 4) was found to be stable following storage at -70°C for at least two weeks. The K_m value for OMP for this form of ODCase, determined on 3 pooled fractions containing a maximum specific activity of 120 nmoles/mg protein/h, was calculated to be 0.9 $\pm$ 0.08 µm.

The heat stability of this form ODCase at 55°C was determined using the same protocols as above except that the protein enzyme was 16 µg in each assay tube of a total volume of 0.5 ml. It was found to be more stable than the crude solubilized ODCase, retaining 75% and 65% of activity following preincubation for 5 and 10 min at 55°C. The intact glycosomal ODCase retained 90% and 80% activity, respectively, under the same conditions.

A single diffuse protein band was observed following polyacrylamide gel electrophoresis of the ODCase eluted from Sepharose CL-6B column chromatography compared to several bands obtained from the crude solubilized enzyme(s).

Discussion

Though apparently membrane bound in all members of the Kinetoplastida, neither of the enzymes OPRTase and ODCase has been previously extracted from their glycosomal environment. Preliminary experiments with the enzymes from *C. luciliae* confirmed that they were strongly bound to the glycosomal membrane. Of the agents tested in attempts at solubilization only Triton-X and CHAPS gave significant recoveries. The former was the better, though the presence of cryoprotectants was essential for reasonable yields (20-30%) to be obtained. By contrast, hexokinase, as for other glycolytic enzymes[2,11] was relatively easily released by Triton.

There was no effect on the K_m of orotate for OPRTase or on the V_{max} of the enzyme after solubilization, suggesting the absence of a barrier for orotate to the enzyme

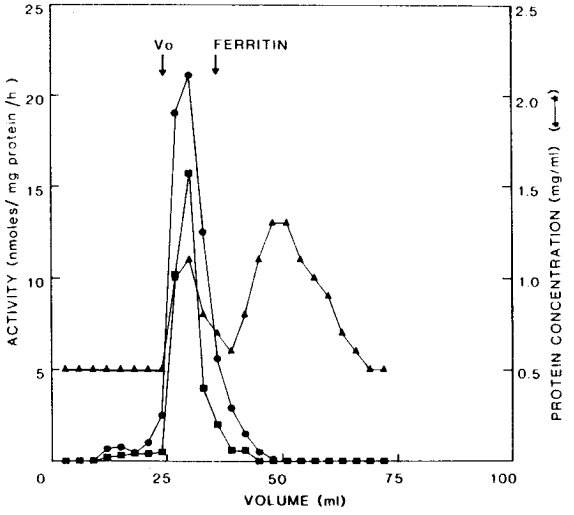

Figure 3. Elution of solubilized OPRTase (■——■). and ODCase (●——●) on Sepharose CL-6B in the presence of 0.25 M sucrose and 20 mM potassium phosphate, pH 7.4 (see Materials and Methods). The positions of the void volume and of the molecular weight marker, ferritin (MW 440,000), are indicated. Recoveries of activity were 30% and 50% for OPRTase and ODCase, respectively.

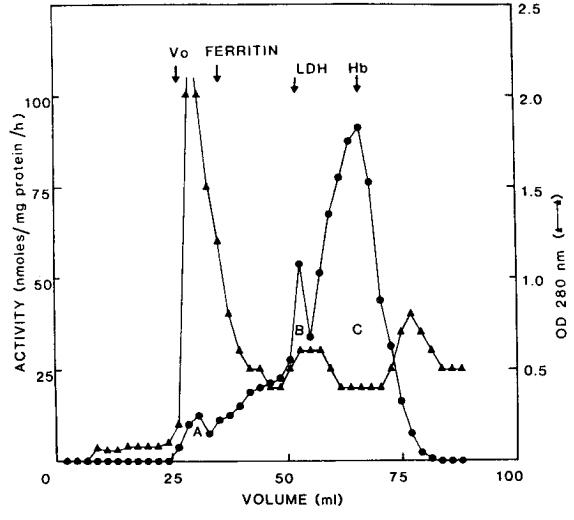

Figure 4. Elution of solubilized OPRTase and ODCase (●——●) on Sepharose CL-6B in the presence of 0.15 M NaCl and the absence of sucrose. Other conditions were as for Fig. 3. The positions of the molecular weight markers, ferritin (MW 440,000), lactate dehydrogenase (MW 136,000), and haemoglobin (MW 65,000) are indicated. Total recovery of activity of ODCase was 62%, of which two thirds was in peak C, the lower molecular weight form (approx. 65,000). The specific activity of pooled fractions of peak C was 120 nmole/mg protein/h. No significant activity of OPRTase was recovered.

active site in the intact glycosome[9]. Similar conclusions were reached for OPRTase in *T. cruzi*[11]. However, the K_m of OMP for the solubilized ODCase (1.66 ± 0.37 μM) was significantly decreased compared to the value obtained for the enzyme in the intact glycosome (viz. 7.5 ± 1.7 μm). This observation is consistent with increased access of exogenous OMP to the ODCase active site, as indicated by the 10-fold decrease in the degree of substrate channelling between the two enzymes following solubilization[9]

Other differences were observed for the solubilized OPRTase and ODCase with respect to the intact glycosomal enzymes. Notably, both enzymes were much more heat labile, indicating a protective role of the membrane. Further there was significant differences in the activation energies of the solubilized with respect to the glycosomal enzyme complex.

The possibility that the solubilized OPRTase-ODCase enzymes were still associated as a complex was supported by the fact that they coeluted from Sepharose CL-6B in a low ionic strength medium (Fig. 3). It has been established that they are on the same polypeptide chain in mammalian cell lines[14,15]. However, the elution pattern varied according to the conditions and in the presence of high salt, at least two and sometimes three peaks of ODCase activity were observed, corresponding to proteins of molecular weights of approximately 500,000, 140,000 and 65,000 respectively (Fig. 4). Under these conditions, OPRTase activity could not be detected and appeared to have been completely lost during the elution process. Though a considerable degree of purification of ODCase appeared to have been achieved this was offset by intrinsic loss of activity of the enzymes.

The presence of OPRTase-ODCase of different molecular weight forms has been observed in other systems. "Complex U", the polypeptide containing both activities, from Ehrlich Ascites carcinoma cells was shown to exist as a monomer-dimer system with molecular weights of 60,000 and 120,000[15]. Brown *et al.* observed three molecular weight species, 62,000, 115,000 and 250,000, respectively, for the OPRTase-ODCase system from human erythrocytes[16]. Multiple molecular weight forms in mouse liver were found by Reyes and Guganig[17] and Campbell *et al.*[18] noted different molecular weight forms in extracts from human liver.

Consistent with these observations, it is possible that for the *C. luciliae* enzyme, the activity of ODCase, which was associated with the protein of molecular weight 65,000, may represent the monomeric form of "Complex U", the OPRTase activity having been lost during the procedure because of its instability. The greater lability of OPRTase compared to ODCase has been reported a number of times in different tissues[16-20] Once freed from the membrane environment, it appears that ODCase (with or without OPRTase activity) also has the ability to form higher molecular weight species.

The glycosome has a number of unusual features. Apart from most enzymes of the glycolytic pathway and OPRTase and ODCase, it also contains the purine salvage enzyme hypoxanthine phosphoryltransferase, enzymes of glycerol metabolism, CO_2 fixation and etherlipid synthesis[21,22]. Most of these enzymes demonstrate a considerable degree of latency; treatment with Triton X-100 results in considerable increase in *in vitro* activity, reflecting enzyme release and/or increased access of low molecular weight substrates[1,22]. The OPRTase-ODCase system is an exception. No latency is observed and it would appear that the enzymes are tightly bound to the glycosomal membrane, though the substrates of OPRTase appear to have easy access from the cytoplasm[11]. Once freed from the membrane environment, the *C. luciliae* OPRTase and ODCase appear to have similar properties to these enzymes from other eukaryotic systems. The possibility that they remain associated with the one polypeptide chain remains to be established.

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บทคัดย่อ

OPRTase และ ODCase เอ็นไซม์สองตัวสุดท้ายในการสร้าง pyrimidine โดยกระบวนการ de novo ในพาราไซท์ของยุงและแมลงวันชื่อ *Crithidia luciliae* ซึ่งอยู่ติดกับผนังของ "ไกลโคโซม" นั้น ถูกสกัดออกมาจาก membrane ได้ โดยใช้ Triton X-100 และสารช่วยรักษาสภาวะเอ็นไซม์ จึงทำให้ได้เอ็นไซม์ถูกสกัดออกมามากกว่า 20% และเมื่อนำส่วนที่สกัดได้ไปผ่าน Sepharose CL-6B พบว่า ODCase มีน้ำหนักโมเลกุลได้หลายฟอร์ม แต่ฟอร์มที่เล็กที่สุดซึ่งถือว่าเป็นโมโนเมอร์นั้น มีน้ำหนักโมเลกุลประมาณ 65,000 ส่วน OPRTase เสียสภาพไปในระหว่างกระบวนการโครมาโตกราฟี