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PURIFICATION OF L-KYNURENINE 3-HYDROXYLASE BY AFFINITY CHROMATOGRAPHY

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SUMMARY

NADP immobilized on agarose is able to adsorb L-kynurenine 3-hydroxylase. The enzyme is released from the adsorbent by passage of a buffer containing 0.5 m*M* NADP through the column. L-Kynurenine 3-hydroxylase was purified 26-fold with a yield of 12% from mitochondrial outer membrane with a procedure involving DEAE-Sepharose CL-6B, Sephacryl S-200 chromatography and NADP-agarose affinity chromatography. This monooxygenase was a homogeneous protein, giving a monomeric molecular weight of 145,000, which had neither any significant NADPH diaphorase activity nor cytochrome b_5-like haem protein. However, the enzyme did not show affinity for a column with L-kynurenine coupled to the gel with a suitable spacer group, and AMP did not serve as an effective ligand in an affinity resin.

INTRODUCTION

We have previously reported a partial characterization of L-kynurenine 3-hydroxylase (EC 1.14.1.2) purified by procedures including gel and ion-exchange chromatography[1]. However, there is no established procedure for obtaining sufficient amounts of the homogeneous enzyme. Owing to the instability of the solubilized enzyme, the conventional purification procedures give very low yields and low purification factors. The lability of this enzyme led us to consider the use of a bio-selective adsorbent. The technique of affinity chromatography is currently used not only for the purification of proteins but also for many other biochemical studies, *e.g.* protein–ligand interactions. Oleson and co-workers[2,3] prepared NADP-agarose for the affinity chromatography of tobacco extracellular nuclease, and they showed that NADP-agarose could bind many nucleases.

One of the common approaches in the preparation of an affinity resin is the immobilization of substrate (L-kynurenine or NADP) for L-kynurenine 3-hydroxylase. NADP-agarose adsorbs L-kynurenine 3-hydroxylase, whereas the monooxygenase does not associate on a column with L-kynurenine coupled to activated CH-Sepharose 4B or epoxy-activated Sepharose 6B. This paper describes the successful use of NADP-agarose affinity chromatography for the purification of this enzyme.

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MATERIALS AND METHODS

NADP-agarose (NADP-adipic acid dihydrazide-agarose, 2.5 μmoles of NADP per ml of wet gel) was purchased from PL Biochemicals (Milwaukee, Wisc., U.S.A.). DEAE-Sepharose CL-6B, Sephacryl S-200, CNBr-activated Sepharose 4B, activated CH-Sepharose 4B, epoxy-activated Sepharose 6B and 5'-AMP-Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden). The coupling reaction of L-kynurenine to the activated Sepharose was performed as described by Cuatrecasas *et al.*[4–7]. L-Kynurenine coupled to the gel was determined spectrophotometrically by measuring the absorbance on the basis of a millimolar extinction coefficient increment of 4.50 $mM^{-1}\,cm^{-1}$ at 360 nm[8,9]. The contents of L-kynurenine (μmole) used for the coupling reaction (A) and free L-kynurenine (μmole) found in the washing buffer (B) are measured. The difference (A — B) is assumed to be the amount of the ligand bound to the gel.

The solubilization of L-kynurenine 3-hydroxylase from mitochondrial outer membrane of rat liver was performed as described previously[1], and this enzyme was partially purified on DEAE-Sepharose CL-6B and Sephacryl S-200 column chromatography, as developed by Nisimoto *et al.*[1,10]. The partially purified sample was used as the enzyme source for affinity chromatography.

L-Kynurenine 3-hydroxylase activity was measured spectrophotometrically at 25°, the rate of NADPH oxidation being estimated on the basis of the decrease in absorbance at 340 nm due to the appearance of 3-OH-L-kynurenine, as described previously[1]. In parallel with the spectrophotometric measurements, the uptake of molecular oxygen depending on the hydroxylation of L-kynurenine was also measured. One unit is defined as that amount of enzyme which catalyses the oxidation of 1 μmole of NADPH per minute at 25° under the assay conditions. The activity of NADPH diaphorase was assayed by the method described by Beutler[11].

Protein was determined by the method of Lowry *et al.*[12] with bovine serum albumin as standard. In some cases, absorbance at 280 nm was measured to determine relative protein contents. For the detection and determination of cytochrome b_5-like haem protein in each purification step, dithionite-reduced minus oxidized difference spectra were measured. Thus, their contents were calculated from the difference spectra on the basis of a molar extinction coefficient increment of 185 $mM^{-1}\,cm^{-1}$ between 423 and 409 nm[13]. In chromatographic experiments, the absorbance at 413 nm of the oxidized state was measured to express relative cytochrome b_5-like haem protein content.

Disc electrophoresis was carried out as previously described[14,15]. SDS-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn[16], at a constant current of 8 mA per tube for 6 h.

RESULTS

Purification of L-*kynurenine 3-hydroxylase by NADP-agarose affinity chromatography*

L-Kynurenine 3-hydroxylase from the mitochondrial outer membrane of rat liver was prufied by isoelectric focusing, but not to a homogeneous state: the purified preparation contained an appreciable amount of cytochrome b_5-like haem protein, based on the absorption spectrum[10].

L-Kynurenine 3-hydroxylase partially purified on DEAE-Sepharose CL-6B and Sephacryl S-200 column chromatography still showed the presence of mitochondrial cytochrome b_5-like haemprotein. Affinity chromatography of the partially purified L-kynurenine 3-hydroxylase on NADP-agarose produced two peaks of the enzyme (Fig. 1). The peak B (tubes NO 26-31), eluted by 50 m*M* Tris–acetate buffer, pH 8.0, containing 0.5 m*M* NADP, 10 m*M* KCl, 0.01% digitonin, 0.1 m*M* dithiothreitol (DTT) and 0.2 *M* mannitol, was collected and concentrated as the purified L-kynurenine 3-hydroxylase, an achievement that the earlier conventional purification procedure failed to accomplish. The first protein peak (peak A), corresponding to the void volume, showed the presence of the haemprotein, and had a very low L-kynurenine 3-hydroxylase activity. As shown in Table I, under the conditions used about 41% of the initial enzymatic activity was bound to NADP-agarose, and AMP could not serve as an effective ligand in affinity resin. The ionic strength and pH of the adsorption buffer had an effect on the binding of L-kynurenine 3-hydroxylase to NADP-agarose. The specific activity and recoveries at each stage of purification are given in Table II. The specific activity of this enzyme increased in parallel with the development of purification process. However, a perfect protective effect was not expected from the addition of mannitol and DTT as described previously, although the addition of these reagents gave significant protection of this enzyme against inactivation[1]. A 26-fold increase in the specific of L-kynurenine 3-hydroxylase, which did not involve mitochondrial cytochrome b_5-like haem protein, was noted with 12% recovery of the initial activity. The value of the specific activity almost corresponded with that of our purified enzyme obtained by the previous method. It was also apparent that L-kynurenine 3-hydroxylase did not possess NADPH diaphorase activity.

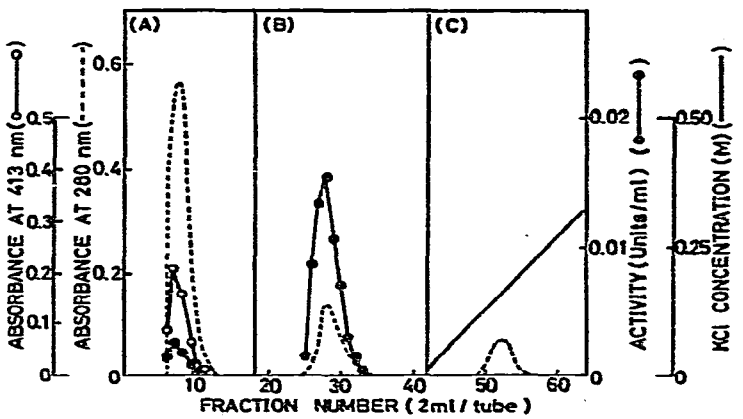

Fig. 1. Elution profile of L-kynurenine 3-hydroxylase from NADP-agarose. Partially purified L-kynurenine 3-hydroxylase (2.0 ml, 7.5 mg as protein) was applied to a column (0.9 × 13 cm) packed with NADP-agarose which had been washed well with adsorption buffer, 50 m*M* Tris–acetate buffer, pH 8.0, containing 10 m*M* KCl, 0.1 m*M* DTT, 0.01% digitonin and 0.2 *M* mannitol. Elution was performed with adsorption buffer (A), 0.5 m*M* NADP in adsorption buffer (B), and a linear gradient of KCl concentration (C) at a flow-rate of 20 ml/h. Fractions were collected and assayed for L-kynurenine 3-hydroxylase. The other procedure is given in the text.

TABLE I

BINDING OF L-KYNURENINE 3-HYDROXYLASE TO VARIOUS ADSORBENTS

The amount of ligand (L-kynurenine) coupled to the resin was estimated as described in Materials and methods. The "Binding" values represent the percentage of applied enzyme activity that was released from adsorption buffer. NADP (0.5 m*M*) was not present (*a*) or present (*b*) in applied enzyme and adsorption buffer, 50 m*M* Tris–acetate buffer, pH 8.0, containing 10 m*M* KCl, 0.1 m*M* DTT, 0.01% digitonin and 0.2 *M* mannitol.

Adsorbent	*Coupled ligand concentration (μmol/ml gel)*	*Binding (%)*
1,6-Diaminohexane–Sepharose 4B Sepharose–NH–$(CH_2)_6$–NH_2	AMP (2.5–6.0)	0.0
Adipic acid dihydrazide–agarose Sepharose–NH–NH–CO–$(CH_2)_4$–CO–NH–NH_2	NADP (2.5)	41.4
Epoxy-activated Sepharose 6B Sepharose–O–CH_2–CH(OH)–CH_2–O–$(CH_2)_4$–O–CH_2–CH(O)–CH_2	L-Kyrurenine (7.5)	0.0[a] 11.5[b]
Activated CH–Sepharose 4B Sepharose–NH–$(CH_2)_5$–C(=O)–O–N(succinimide)	L-Kynurenine (14.8)	0.0
Cyanogen bromide–activated Sepharose 4B Sepharose–O–C(=NH)–O	L-Kynurenine (15.4)	0.0
Sepharose 4B	L-Kynurenine (0.1)	0.0

Affinity chromatography of L-*kynurenine 3-hydroxylase on* L-*kynurenine-coupled Sepharose*

As shown in Table I, the partially purified L-kynurenine 3-hydroxylase did not show affinity for a column with L-kynurenine coupled to activated Sepharose with a suitable spacer group. When 0.5 m*M* NADP was added to the partially purified enzyme solution and adsorption buffer, as shown in Fig. 2, a portion of the monooxygenase (protein peak B) was associated with L-kynurenine coupled to epoxy-activated Sepharose 6B, and it could be eluted by a linear gradient change of ionic strength. It is reasonable to suggest that an appropriate conformational change is induced in the monooxygenase around the active site for interaction with NADP, *i.e.* an increase of the reactivity toward L-kynurenine fixed on epoxy-activated Sepharose in the presence of NADP. Such low efficiency has not been observed on the association of L-kynurenine-bound Sepharose with other spacer groups. A particular length of spacer group on the gel is presumably required for adsorption of the enzyme. However, this affinity chromatography was found not to be effective for a purification of this enzyme because pronounced inactivation occurred under the given operating conditions.

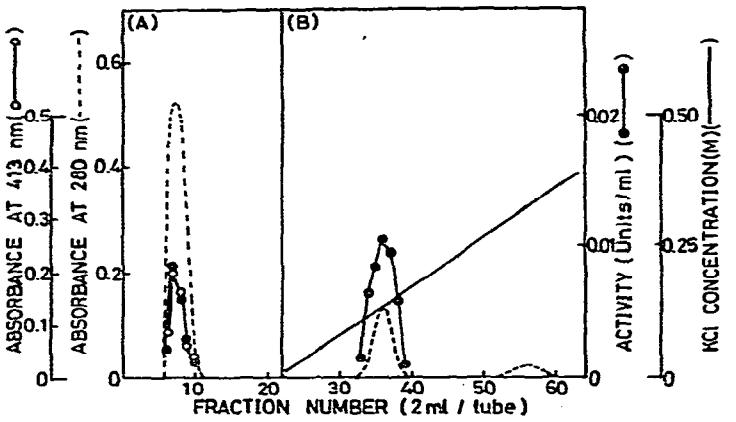

Fig. 2. Affinity chromatography of L-kynurenine 3-hydroxylase on L-kynurenine coupled to epoxy-activated Sepharose 6B. Partially purified L-kynurenine 3-hydroxylase from Sephacryl S-200 column chromatography (2.0 ml, 7.5 mg as protein) was applied to a column (0.9 × 13 cm) packed with L-kynurenine-bound epoxy-activated Sepharose 6B which had been equilibrated well with 50 m*M* Tris–acetate buffer, pH 8.0, containing 10 m*M* KCl, 0.1 m*M* DTT, 0.01% digitonin, 0.2 *M* mannitol and 0.5 m*M* NADP. Elution was performed with the same buffer as equilibration (A) and a linear gradient of ionic strength (B) at a flow-rate of 20 ml/h. Fractions were collected and assayed for L-kynurenine 3-hydroxylase.

SDS-polyacrylamide gel electrophoresis

As shown in Fig. 3, solubilized L-kynurenine 3-hydroxylase was usually contaminated by several protein components as indicated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. The monooxygenase isolated by NADP-agarose affinity chromatography is apparently homogeneous on disc and SDS-polyacrylamide gel electrophoresis, and the monomeric molecular weight of this enzyme was determined from the calibration curve as *ca.* 145,000.

DISCUSSION

Methods for purifying enzymes using an affinity column of agarose-bound NADP have recently been reported[17;18]. Yoshida[19] reported that human glucose 6-phosphate dehydrogenase (EC 1.1.1.48) associated with NADPH was effectively bound with agarose-bound NADP, whereas the enzyme associated with NADP was poorly bound with agarose-bound NADP. The application of NADP bound to Sepharose hydrazide for the absorption of glucose 6-phosphate dehydrogenase has been described by Lamed *et al.*[20]. These studies all demonstrate that Sepharose-nucleotide and Sepharose-NADP columns can be used for the purification by affinity chromatography of enzymes to which these compounds are substrate, coenzymes and effectors. As affinity chromatography is the most specific and efficient method for enzyme purification, it is desirable to use it for the purification of L-kynurenine 3-hydroxylase. Thus, it was necessary to use a relatively pure preparation for successful affinity chromatography with a minimum duration between the desalting process and the elution from the NADP-agarose column, in addition to protection against de-activation. However, a greater portion of the enzyme is expected to be deactivated

TABLE II

PURIFICATION OF L-KYNURENINE 3-HYDROXYLASE FROM THE MITOCHONDRIAL OUTER MEMBRANE OF RAT LIVER

The enzyme assay and the determination of cytochrome b_5-like haem protein were performed as described in the text.

Step	*Total volume (ml)*	*Protein (mg)*	*L-Kynurenine 3-hydroxylase*		*Purification factor*	*Cytochrome b_5-like haem protein*	
			Specific activity (nmoles/min·mg protein)	*Recovery (%)*		*Specific content (nmoles/mg protein)*	*Recovery (%)*
1 Outer membrane	62	9.0	2.8	100	1	0.288	100
2 Digitonin solubilization	22	13.0	9.8	85.7	3.5	0.310	61
3 DEAE-Sepharose CL-6B eluate	21	3.25	28.5	60.1	10.2	0.257	21
4 Sephacryl S-200 eluate	7.6	3.75	33.6	29.6	12.0	1.88	8.7
5 NADP-agarose eluate	2.0	2.55	73.0	11.5	26.1	0.0	0.0

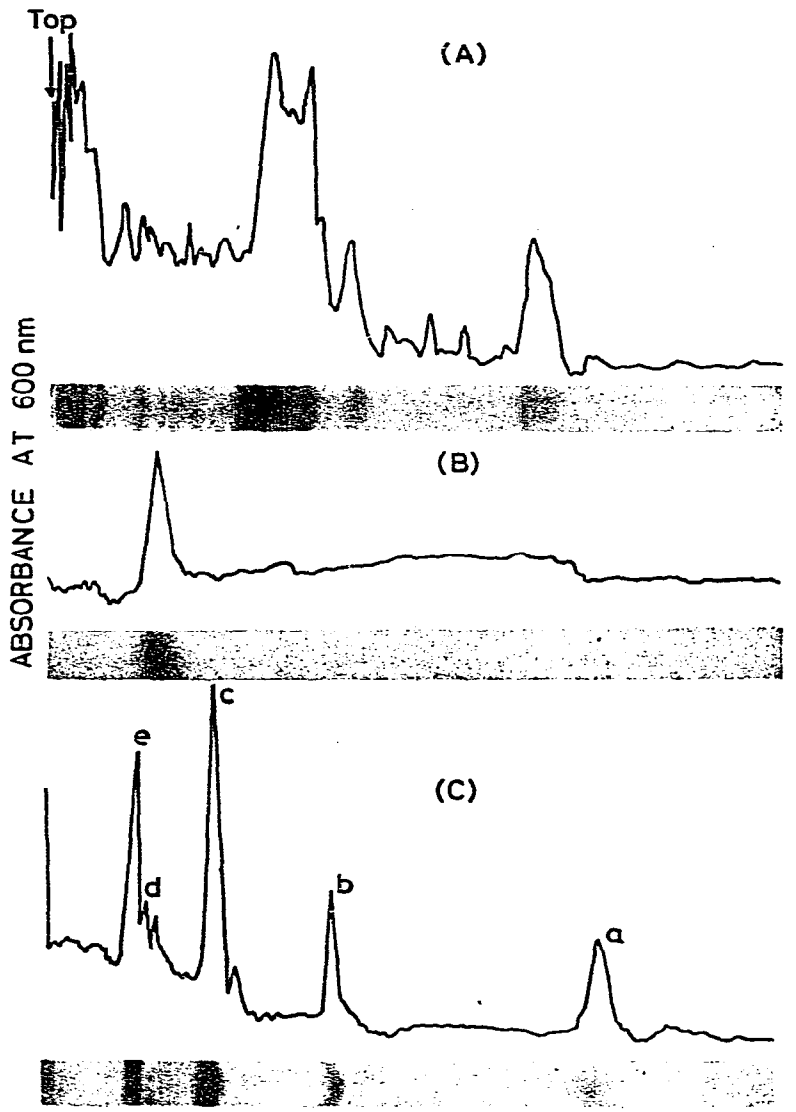

Fig. 3. SDS-gel electrophoresis of proteins solubilized from mitochondrial outer membrane and purified L-kynurenine 3-hydrolylase. About 150 μg protein of solubilized outer membrane fraction (A) and *ca.* 15 μg protein of purified L-kynurenine 3-hydroxylase (B) were applied to 10% polyacrylamide gel columns containing 1% SDS. The proteins used as standards (C), with their abbreviations, are as follows: a = trypsin inhibitor; b = α-subunit of the RNA polymerase; c = bovine serum albumin; d = β-subunit of the RNA polymerase; e = β'-subunit of the RNA polymerase. Their molecular weights were assumed to be 21,000, 39,000, 68,000, 155,000 and 165,000, respectively. The gels were scanned at 600 nm.

during the purification procedure using 50 m*M* Tris–acetate buffer, pH 8.0, containing 0.1 m*M* DTT, 0.01% digitonin and 0.2 *M* mannitol. Therefore, the best practical method of stabilizing the monooxygenase in affinity chromatography remains to be found.

L-Kynurenine 3-hydroxylase can be purified by isoelectric focusing, but not to a homogeneous state: our purified preparation was contaminated with cytochrome b_5-like haem protein bound with the mitochondrial outer membrane of rat liver[1,10]. In fact, it was necessary to establish whether this haem protein in the purified preparation takes part in L-kynurenine hydroxylation, or whether it is an impurity which cannot easily be separated from the enzyme. We obtained a homogeneous enzyme which had neither any significant NADPH diaphorase activity nor this haem protein. In contrast to the previous report[10], the results were satisfactory with respect homogeneity, overall yield and the simplified purification.

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