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Cell-free synthesis of alkaline lipase, a glyoxysomal membrane protein, from castor bean endosperm

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Polyadenylated RNA from castor bean endosperm was translated in a wheat germ cell-free protein synthesis system, and alkaline lipase, an integral glyoxysomal membrane protein, was immunoprecipitated. The apparent molecular mass of the lipase synthesized in vitro was slightly higher than that of the mature enzyme (62 kDa). When mRNA derived from free and membrane-bound polysomes in the endosperms was translated in vitro, the lipase was predominantly recovered from the products of the free polysomal mRNA, suggesting that the membrane protein is post-translationally inserted into the membrane. The amino-terminal 8 amino acid residues of the mature lipase were sequenced.

Glyoxysome biogenesis; Membrane protein; Glyoxysomal lipase; Translation; Enzyme precursor; (Castor bean)

1. INTRODUCTION

In recent years, much information about the mechanism of microbody biogenesis, especially the synthesis and transport of proteins into the organelle, has been accumulated, and the peripheral membrane proteins and matrix proteins in microbodies have been shown to be synthesized on free polysomes and post-translationally imported into the organelle [1,2]. However, few reports are available concerning the synthesis and insertion of microbody integral membrane proteins except for a rat liver peroxisomal protein [3]. We have directed our attention to the synthesis of microbody membrane proteins in order to understand the mechanism of biogenesis of microbody membrane.

Active biogenesis of glyoxysomes takes place in the endosperm of germinating castor bean [4], and the biogenesis is accompanied by active formation of alkaline lipase, which is the only known enzymically active membrane protein in the glyoxysomes [5]. The lipase (62 kDa) has been concluded to be an integral membrane component because it is not solubilized with high salt concentrations nor low concentrations of deoxycholate [5,6]. In the present communication, we suggest that the lipase is post-translationally inserted into the glyoxysomal membrane.

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2. MATERIALS AND METHODS

2.1. *Plant material*

Dry castor bean (*Ricinus communis* L.) seeds were soaked for 24 h and germinated in moist vermiculite in darkness at 30°C. After germination for 3 days, the endosperm tissue was taken from uniformly germinating seedlings.

2.2. *Enzyme purification and antibody preparation*

The glyoxysomal lipase was purified from castor bean endosperm and antibodies were raised in a rabbit against the purified lipase as described previously [6]. The IgG fraction was obtained from the antiserum by ammonium sulfate frac-

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tionation and DEAE-cellulose column chromatography.

2.3. *Poly(A)$^+$ RNA preparation and cell-free translation*

Poly(A)$^+$ RNA was prepared from the 3-day-old endosperms by extraction with a mixture of phenol/chloroform/isoamylalcohol and oligo-(dT)-cellulose column chromatography [7]. The translation in a wheat germ extract was carried out at 25°C for 60 min using 20 μl of a reaction mixture containing 10 μCi [^{35}S]methionine (1000 Ci/mmol) [7]. The immunoprecipitate from the in vitro synthesized proteins was subjected to SDS-polyacrylamide gel electrophoresis and the protein bands were visualized by fluorography.

2.4. *Amino acid sequence analysis*

The glyoxysomal lipase was solubilized from the membrane with deoxycholate [6] and purified by SDS-polyacrylamide gel electrophoresis. Sequential preparation of phenylthiohydantoin derivatives of amino acids from the N-terminal part and analysis of the derivatives produced were performed in a JEOL JAS-47K sequence analyser [8].

2.5. *Other methods*

The specificity of the antibodies was tested by immunoblot analysis [9] using horseradish peroxidase-linked protein A (Amersham). SDS-polyacrylamide gel electrophoresis on a 12% polyacrylamide slab gel was performed according to Laemmli [10]. Protein concentration was determined by the method of Lowry et al. [11]. In vivo labelling of endosperm proteins and preparation of free and membrane-bound polysomes from the endosperms were carried out according to Roberts and Lord [12].

3. RESULTS AND DISCUSSION

The antibodies prepared in the present work precipitated alkaline lipase specifically from total membrane proteins in castor bean endosperm glyoxysomes (fig.1). In this study, we used endosperms from 3-day-old castor bean seedlings, because the rate of lipase synthesis had been observed to be the highest at the 3rd day after the onset of germination (Maeshima, M. et al., unpublished).

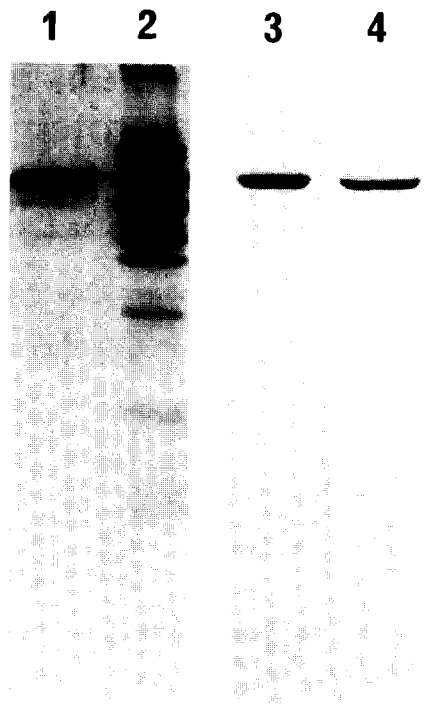

Fig.1. Immunoblot analysis of the specificity of anti-(glyoxysomal lipase) antibodies. Lanes: 1 and 2, gel stained with Coomassie brilliant blue; 3 and 4, immunoblot with anti-(glyoxysomal lipase) IgG and horseradish peroxidase-linked protein A. Lanes: 1 and 3, the purified glyoxysomal lipase (6 μg); 2 and 4, the glyoxysomal membrane (25 μg).

Next we compared the in vitro and in vivo synthesized glyoxysomal lipase on the SDS-polyacrylamide gel (fig.2, lanes 1 and 2). When poly(A)$^+$ RNA from the endosperms was translated in vitro in the wheat germ protein synthesis system and the translation products were immunoselected with anti-(glyoxysomal lipase) IgG, a major and several minor labelled polypeptides were detected on the polyacrylamide gel (fig.2, lane 2). The major polypeptide, but not the others, was not immunoprecipitated with anti-(glyoxysomal lipase) IgG preincubated with the purified enzyme (fig.2, lanes 3 and 4), indicating that the major polypeptide immunoprecipitated with the antibodies is the glyoxysomal lipase. This polypeptide was slightly larger than the lipase labelled in vivo in the 3-day-old endosperms (fig.2, lanes 1 and 2), suggesting that at least several amino acid

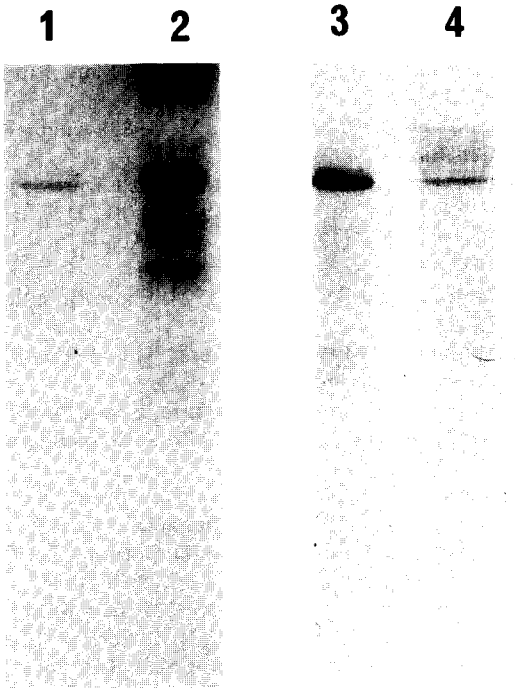

Fig.2. Comparison between the in vitro and in vivo synthesized glyoxysomal lipase. Lane 1: 3-day-old endosperms were incubated with [^{35}S]methionine for 2 h by the method of Roberts and Lord [12]. After preparation of the crude glyoxysomal fraction, the glyoxysomal lipase was immunoselected with anti-(glyoxysomal lipase) IgG and protein A-Sepharose from the fraction. Lane 2: poly(A)$^+$ RNA from 3-day-old endosperms was translated in a wheat germ cell-free protein synthesis system and the lipase was immunoprecipitated from the total translation product. Lanes 3 and 4: immunoprecipitates from the in vitro synthesized proteins with anti-(glyoxysomal lipase) IgG preincubated without and with the purified lipase, respectively. In vitro and in vivo labelled polypeptides were analysed by SDS-polyacrylamide gel electrophoresis and fluorography.

residues may be eliminated in situ from the newly synthesized polypeptide to form the mature enzyme. The amino-terminal sequence of the isolated glyoxysomal lipase was as follows.

1 2 3 4 5 6 7 8

Asp-Gly-Ala-Pro-Ile-Lys-Lys-Pro-

At present the structure of the cleavable extra peptide of the lipase is unclear, and we are now trying to clone the cDNA for glyoxysomal lipase mRNA to deduce the amino acid sequence of the extra peptide from its nucleotide sequence.

The soluble proteins in microbodies such as isocitrate lyase have been shown to be synthesized on free polysomes [1,2,12]. On the other hand, the integral membrane proteins of glyoxysomes have been suggested to be synthesized on membrane-bound polysomes [1]. To determine whether the glyoxysomal lipase is synthesized on free or membrane-bound polysomes, both total or poly(A)$^+$ RNAs were prepared from the free and membrane-bound polysomes of the 3-day-old endosperms and translated in a wheat germ cell-free protein synthesis system, and the radioactivity in the immunoprecipitate from the translation products with anti-(glyoxysomal lipase) IgG was assayed (table 1). The labelled glyoxysomal lipase was predominantly recovered from the translation products of free polysomal RNA, which strongly suggests that the glyoxysomal lipase may be synthesized on free polysomes.

Table 1

In vitro synthesis of the glyoxysomal lipase with mRNA derived from free and membrane-bound polysomes

Polysomes	RNA extracted (μg)	[^{35}S]Met in glyoxysomal lipase	
		10^3 cpm/μg RNA	10^3 cpm
Total RNA			
Free	1370	1.49	2040
Bound	235	0.731	172
Poly(A) rich RNA			
Free	11.0	75.3	828
Bound	2.30	22.3	51.3

Free and membrane-bound polysomes were prepared from about 30 g of 3-day-old endosperms, and in vitro translation was carried out with 2 μg and 0.2 μg of total RNA and poly(A)$^+$ RNA, respectively. Each immunoprecipitate was electrophoresed on the SDS-polyacrylamide gel and visualized by fluorography, then the proportion of the label in the lipase to the total in the immunoprecipitate was determined from densitometric scanning of the fluorogram. The radioactivity in the lipase was calculated from the proportion and the radioactivity of an aliquot of the immunoprecipitate

In conclusion, we propose that the glyoxysomal lipase is synthesized in a form with a short cleavable extra peptide on free polysomes and post-translationally inserted into the glyoxysomal membrane. A similar proposition has been made for the 22-kDa integral membrane polypeptide in rat liver peroxisomes except that the polypeptide is synthesized in vitro in a form lacking a cleavable extra peptide [3]. At present the function of the extra peptide for the lipase remains to be investigated.

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REFERENCES

[1] Trelease, R.N. (1984) Annu. Rev. Plant Physiol. 35, 321–347.
[2] Lazarow, P.B. and Fujiki, Y. (1985) Annu. Rev. Cell Biol. 1, 489–530.
[3] Fujiki, Y., Rachubinski, R.A. and Lazarow, P.B. (1984) Proc. Natl. Acad. Sci. USA 81, 7127–7131.
[4] Beevers, H. (1982) Ann. NY Acad. Sci. 386, 243–253.
[5] Muto, S. and Beevers, H. (1974) Plant Physiol. 54, 23–28.
[6] Maeshima, M. and Beevers, H. (1985) Plant Physiol. 79, 489–493.
[7] Hattori, T., Iwasaki, Y., Sakajo, S. and Asahi, T. (1983) Biochem. Biophys. Res. Commun. 113, 235–240.
[8] Bhown, A.S., Mole, J.E., Weissinger, A. and Bennett, J.C. (1978) J. Chromatogr. 148, 532–535.
[9] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[10] Laemmli, U.K. (1970) Nature 227, 680–683.
[11] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[12] Roberts, L.M. and Lord, J.M. (1982) Eur. J. Biochem. 119, 43–49.