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ENTRAPMENT OF PHENYLALANINE AMMONIA-LYASE IN SILK FIBROIN FOR PROTECTION FROM PROTEOLYTIC ATTACK*

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Phenylalanine ammonia-lyase was entrapped in silk fibroin. The entrapped enzyme showed a similar Km for Phe and pH optimum to the free enzyme. It was resistant against chymotrypsin and trypsin in vitro. To assess the activity in vivo, the free or entrapped enzymes and then Phe were injected into rat duodenum, and cinnamate, a product, in plasma was determined as the most direct evidence of the enzyme activity. The entrapped enzyme but not the free form caused a marked raise of plasma cinnamate. It declined with a half life of about 45 min, which was significantly longer than that(10 - 15 min) observed upon i.v. administration of cinnamate. These results indicated that the entrapped enzyme was actively degrading Phe in the intestinal tract. Entrapment of phenylalanine ammonia-lyase in fibroin thus provides a new prospect for oral enzyme therapy of phenylketonuria.

Phenylketonuria(PKU) is the result of a genetic deficiency of Phe hydroxylase (EC 1.14.16.1) in the liver, an enzyme that catalyzes the conversion of Phe to Tyr, and is characterized by high levels of circulating Phe and massive urinary excretion of its metabolites(1). Phenylalanine ammonia-lyase(PAL, EC 4.3.1.5), which catalyzes the elimination of ammonia from Phe to form cinnamate, has been one of possible candidates for the treatment of PKU(2-4) because it degrades Phe without any cofactors(5) and the cinnamate formed is non toxic(6), and is obtained easily from yeast(7). Hoskins et al.(3,4) has reported the oral application of PAL to PKU patients, suggesting that orally ingested PAL lowered the circulating Phe levels by degrading Phe released from food digested in the small intestine. However, there is a drawback when administered orally in that PAL is inactivated by chymotrypsin

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and trypsin present in the intestinal tract(8). By chemical modifications of PAL, its susceptibility to such proteases was not altered or increased(8). Recently, permeabilisation of yeast cells containing PAL was proposed for protection of PAL from a proteolytic attack, however, the enzyme level must be increased in the cell for in vivo treatment(9).

To provide protease resistance to PAL, we attempted to prepare an entrapped PAL using natural materials for oral use and were successful in entrapping PAL in a protein, silk fibroin. Here we report that entrapped PAL, but not its free form, is resistant against intestinal proteases in vitro and in vivo, and the technique of entrapment of enzymes in silk fibroin may provide a new prospect for oral enzyme therapies of PKU and other diseases for which a dietary control is essential.

MATERIALS AND METHODS

Chemicals. Trypsin(TypeIII) and α-chymotrypsin(TypeI-S) were purchased from Sigma. All chemicals were reagent grade and used without further purification.
Assay for PAL. PAL activity was assayed as described in (10). The reaction with entrapped PAL was monitored with a dual wave length method at an absorbance rate of ($\lambda_{290\ nm} - \lambda_{350\ nm}$) over time to discount the turbidity. One unit was defined as the amount of enzyme required to catalyze the appearance of 1 μmol of cinnamate per minute at 30°.
Purification of PAL. Rhodotorula glutinis(0559 Institute for Fermentation, Osaka) was cultured as described in (6). The cells obtained(18 g of wet weight per liter of medium) were suspended in two volumes of Tris/HCl buffer (50 mM, pH8.5) and immediately disrupted by a Dyno Mill(Type DMK-15s) followed by a Cepa continuous flow centrifuge to obtain a crude extract. The extract (3 liters) containing 15000 units of PAL which was obtained from 2.4 kg of wet weight of the cells was incubated at 55° for 10 min, following that 4.5 liters of Tris/HCl buffer(50 mM, pH8.5) were added and the extract was centrifuged. The supernatant was concentrated to 900 ml by a hollow fiber(Type DC-10, Amicon). Ammonium sulfate(15% saturation) was added to the concentrated enzyme solution, and the supernatant after centrifugation (10000g x 20 min) was submitted to chromatography on a Phenyl-Sepharose CL-4B column(6 x 35 cm) equilibrated with Tris/HCl buffer(50 mM, pH8.0) containing ammonium sulfate(15% saturation). The column was washed with Tris/HCl buffer(25 mM, pH8.0) containing 7.5% saturation of ammonium sulfate and 25%(V/V) of ethylene glycol, and the enzyme activity was eluted with a gradient of 7.5 to 0% saturation of ammonium sulfate as well as 25 to 50% of ethylene glycol in the same buffer with a flow rate of 4 ml/min. Fractions containing an activity of 2 units/ml or greater were pooled, and the enzyme was concentrated to 280 ml and dialyzed against a potassium phosphate buffer(5 mM, pH6.5). Purified PAL(11000 units) had a specific activity of 2.3 units/mg.
Preparation of silk fibroin. Silk fibroin was refined by treatment at 80° for 3 h in an alkaline solution containing 2.8%(W/W) of soap(95%(W/W) beef tallow) and 0.33%(W/W) of sodium carbonate. After washing with water and allowing to dry, it was solubilized by incubation at 80 - 85° for an hour with 41%(W/W) of ethanol. Solubilized fibroin was completely dissolved with

hot water, filtered, and dialyzed against distilled water before use to obtain 5%(W/V) fibroin solution.

Entrapment of PAL in fibroin. The fibroin solution(49 ml) was mixed with 25.3 ml of purified PAL solution(1000 units). The mixture was added to one liter of cold potassium phosphate buffer(50 mM, pH6.5) containing ammonium sulfate(43% saturation) with vigorous agitation(8000 rpm) for 3 min and allowed to stand at 4° for 30 min. The salted-out fibroin containing PAL was filtered and washed 3 times with 400 ml of cold potassium phosphate buffer(5 mM, pH6.5). The entrapped PAL obtained was suspended in 30 ml of the same buffer and lyophilized overnight. Then, it was passed through a mesh(No. 100) with 149 μm pore size in a chamber which was regulated to provide a 50% relative humidity. The entrapped PAL(2.46 g) with an activity of 110 units/g was obtained and stored at -20°.

Animal studies. Male Sprague-Dawley rats, weighing 170 to 200 g, were housed in a 12-h light-dark cycle with a commercial diet CE-2(Nihon Clea, Tokyo) and water available ad libitum. Abdominal operations were performed under diethyl ether anesthesia. Entrapped or free PAL(4 units/ml of potassium phosphate buffer(50 mM, pH7.2)) was injected into duodenum using a disposable syringe with a 25-gauge needle, then 100 mg/kg of Phe(30 mg/ml of the same buffer) was injected as above. After the injection, the abdominal incision was immediately sutured. Blood(0.5 ml) was taken by cardiac puncture using a heparinized glass syringe with a 23-gauge needle under light diethyl ether anesthesia. The rats were returned to individual cages until next sampling. Plasma was obtained by centrifugation and stored at -20°.

Determination of plasma cinnamate. Plasma cinnamate was determined by HPLC as described in (11). The chromatographic separation was achieved at room temperature on a reverse-phase column of YMC-Pack A-312 ODS(6 mm x 15 cm, Yamamura Chemical Lab. Co., Ltd., Kyoto) at a flow rate of 2 ml/min with an isocratic solvent system(acetonitril/5%(W/V) acetic acid, 35/65, V/V). The eluate was monitored at 290 nm. Under our conditions, the retention time of cinnamate was 5.9 min and the sensitivity was 5 pmol.

RESULTS

Preparation of entrapped PAL in silk fibroin and its enzymological properties.

PAL was purified from yeast as described under "MATERIALS AND METHODS". The enzyme with a high specific activity(2.3 units/mg) was recovered in high yields(73.3%) by a single step hydrophorbic chromatography on a Phenyl-Sepharose CL-4B column.

To provide protease resistance to PAL, we prepared an entrapped PAL using silk fibroin as described under ""MATERIALS AND METHODS". Entrapped PAL(2.46 g) with an activity of 110 units/g was obtained as a white powder with less than 150 μm particle size. Entrapped PAL was more stable than lyophilized free PAL: The remaining activity after 82-days storage at 4° was 75.4 and 34.4% for entrapped and free PAL, respectively. When entrapped or free PAL was incubated at 65° in potassium phosphate buffer(0.1 M, pH7.3), half lives of PAL activity were identical(8.2 min) for both preparations. A Km value of entrapped PAL for Phe at 30° in Tris/HCl buffer(0.1 M, pH8.5) was 0.7

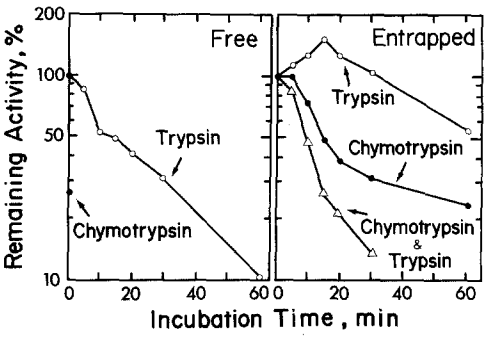

Fig. 1. Protease susceptibility of free(left) or entrapped(right) PAL in vitro. One unit of free or entrapped PAL in 1 ml of Tris/HCl buffer(50 mM, pH8.5) was incubated at 30° and 20 µl of α-chymotrypsin and/or trypsin (5 mg/ml) were added at 0 time. In the case with free PAL, 10 mg of fibroin which were processed the same way as entrapping but containing no PAL were added to the reaction mixture.

to 0.9 mM, which was similar to that of free PAL(10). A pH-activity curve of entrapped PAL between pH6.5 and 9.0 was identical with that of free PAL(data not shown). These results showed that stability in storage was improved without a significant change of enzymological characteristics of PAL

Protease susceptibility of entrapped PAL in vitro. Free PAL was inactivated rapidly when incubated with an excess amount of α-chymotrypsin, whereas it was more resistant against tryptic treatment(Fig. 1, left). In contrast with this, entrapped PAL in fibroin showed a resistance against chymotrypsin or trypsin although PAL inactivation was accelerated to some extent in the presence of both proteases(Fig. 1, right). A transient increase(50% or more) of the activity of entrapped PAL when incubated with trypsin is thought to be due to exposure and release of PAL inside the fibroin particle whose activity could not be estimated before the tryptic treatment. These in vitro results showed that protease resistance was given to PAL by entrapment in silk fibroin.

Absorbance and clearance of cinnamate in rats. To assess the in vivo activity of free or entrapped PAL, we examined whether or not the reaction product, cinnamate, was absorbed into plasma from rat duodenum and how long its level was maintained in plasma. Fig. 2 shows the change of plasma cinnamate level after its duodenal injection. It was well absorbed into blood circulation

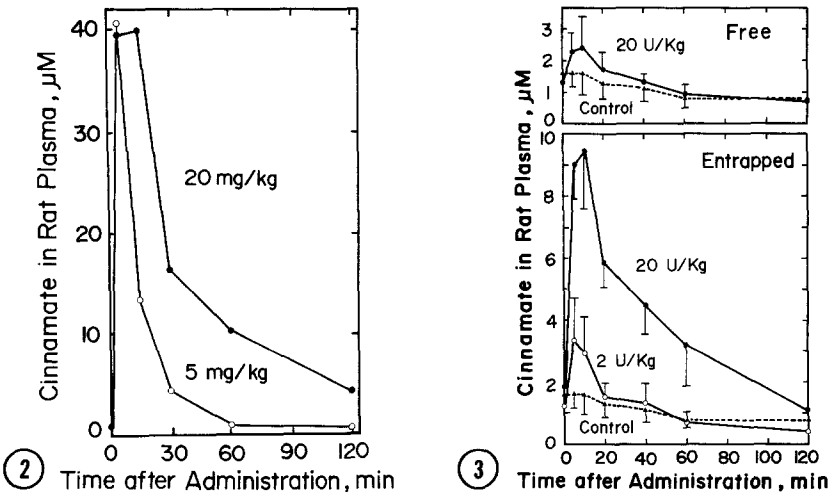

Fig. 2. Absorption and clearance of cinnamate after its duodenal injection. Cinnamate was injected into duodenum of nonfasted rats(○—○, 5 mg/kg; ●—●, 20 mg/kg). Blood(0.5 ml) was taken by cardiac puncture and plasma cinnamate was determined. Each value is the mean of individual 4 experiments.

Fig. 3. Activity of free or entrapped PAL in rat small intestine. Free or entrapped PAL was injected into duodenum of nonfasted rats followed by Phe(100 mg/kg) administration; free PAL(●—●, 20 units/kg), entrapped PAL (○—○, 2 units/kg; ●—●, 20 units/kg), and Phe administration without PAL (▲---▲). Blood(1 ml) was drawn by cardiac puncture and plasma cinnamate was determined. Each value is the mean(S.E.) of individual 4 experiments. Data obtained from sham operated rats(no Phe nor PAL administration) were not shown, but were almost same as those of control(▲---▲).

to give the maximal level after 5 to 10 min and then disappeared rapidly from plasma. A half life on i.v. injection of cinnamate(5 mg/kg) was 10 to 15 min(data not shown). These results suggested that it was possible to estimate the reactivity of PAL in the intestinal tract by the frequent measurement of plasma cinnamate.

Activity of free or entrapped PAL in vivo. When entrapped PAL(2 or 20 units/kg) was administered to rat duodenum followed by injection of authentic Phe(100 mg/kg) as described under "MATERIALS AND METHODS", plasma cinnamate increased significantly, whereas the increase was not significant with free PAL(20 units/kg) administration(Fig. 3). A half life of plasma cinnamate on the administration of entrapped PAL(20 units/kg) was 45 min, suggesting that the entrapped PAL was actively degrading Phe in the intestinal tract. The amount of cinnamate formed by the action of entrapped PAL(20 units/kg) was estimated as about 2 mg for 2 h, taking into account the area under plasma concentration on the duodenal injection of cinnamate(5 mg/kg, Fig. 2). The

basal level of cinnamate with or without PAL treatment was due to cinnamate derived from the commercial diet(data not shown).

DISCUSSION

We successfully prepared herein protease-resistant PAL by entrapping the enzyme in silk fibroin. Entrapped PAL obtained acquired a protease resistance *in vitro*(Fig. 1) as well as stability in storage, while the enzymological properties were well conserved. The determination of plasma cinnamate revealed that entrapped PAL, but not its free form, was actively degrading Phe in the intestinal tract(Fig. 3). Here the fatal drawback of this enzyme's susceptibility to proteases was overcome by entrapment. For the oral application of entrapped PAL to PKU patients, a degree of depletion of circulating Phe must be examined using phenylketonuric animals although there have been no experimental animals specific for PKU.

The method will be applied to other enzymes without much loss of activity. Entrapment of enzymes in fibroin thus provides a new prospect for oral enzyme therapies of PKU and other diseases for which a dietary control is essential.

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