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Surface Activities of Ginseng Saponins and Their Interactions with Biomolecules : (V) Ginseng Saponins Can Be Used in Cytochrome c Isolation

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Abstract□The effects of treating bovine heart mitochondria with potassium chloride and surfactants such as digitonin and n-dodecy-β-maltoside (DMS) including plant saponins on extracting cytochrome c were examined. The spectra given by the cytochrome c-containing solutions from the extraction were inspected to ascertain whether ginseng and bellflower saponins could be used instead of the generally-employed surfactants of digitonin and DMS. These studies implied that the effect of ginseng saponins is superior to that of digitonin but inferior to that of DMS, and give rise to the idea of substitutional property of ginseng saponins for the widely-employed surfactants in the extraction of mitochondrial intermembrane cytochrome c. The substitution for the solubilizing surfactants by bellflower saponins could, however, not presumably be anticipated; while ginseng saponin mixture are a suitable substitute.

Key words□Cytochrome c, mitochondria, ginseng saponin.

Introduction

For the proteins in mitochondrial membranes, a number of researches have long been devoted in connection with ATP synthesis[1] and heat formation.[2] Much knowledge for the internal pathway, that is, the electron transport from NADH or $FADH_2$ through mitochondrial inner membrane only has accumulated in contrast with those for the external pathway involving non-phosphorylating mitochondrial outer membrane,[2,3] cytochrome c, and phosphorylating cytochrome oxidase. The studies on the external pathway, amytal and antimycin A-insensitive one,[3] established the peripheral being for cytochrome c as follows. Mokhova *et al.*[2] proposed a mechanism of the urgent heat production operated for the cold adaptation within the hepatic mitochondria of cold-exposed rats. They interpreted that this mitochondrial mechanism operates through the activation of the external electron transport due to the mobilization or desorption of inner membrane-bound cytochrome c. Bernardi and Azzone[3] described the mitochondrial aerobic oxidation for exogenous NADH which utilizes both NADH-cytochrome b_5 reductase/cytochrome b_5 of outer membrane and cytochrome oxidase of inner membrane. Cytochrome c electron shuttle exists between cytochrome b_5 and cytochrome oxidase in this electron-transport chain. Lee and Lee[4] described in their report from an experiment *in vitro* that ginseng saponin added to the NADH-oxidation system might mobilize the intermembrane cytochrome c of mitochondria and activate the external electron transport which produces heat because of the shortage of ADP phosphorylations.

Membrane-protein purification makes use of detergents for the desorption of the protein from membrane structure.[5] The surfactants used generally for solubilizing the membrane proteins being spanned across or attached to lipid bilayer are n-dodecyl-β-D-maltoside (DMS) or digitonin. Plant saponins which are surface active[6] are supposed to have the ability to mobilize membrane-bound proteins

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and to act as a substitute detergent in place of costly DMS or digitonin. The possibility of solubilizing or desorbing the cytochrome c adherent to the cytosolic face of mitochondrial inner membrane[7] will be examined.

Materials and Methods

1. Materials

Fresh bovine heart was obtained from a local slaughterhouse. Saponins were prepared from Korea White Ginseng and bellflower. Tris (hydroxymethyl)aminomethane, DMS were obtained from the Sigma Chemical Co. Digitonin was purchased from Wako. Sodium dithionite was a product of Yakuri, Osaka.

2. Preparation of bovine heart mitochondria

Mitochondria were prepared after the method of Darley-Usmar *et al.*[8] as follows. Approximately 1 kg of bovine heart from which fat clots were trimmed off was ground and homogenized with a blender and a Teflon-pestle homogenizer in 0.01 M Tris-HCl, pH 7.8 containing 0.25 M sucrose and centrifuged at 1200×g for 20 min. The pH of the supernatant was adjusted to 7.8 with 2 M Tris and recentrifuged at 26000×g for 15 min. The pellet was preserved at −20℃ as the mitochondrial preparation for cytochrome c extraction. The process for obtaining mitochondrial pellet was conducted at from 0 to 4℃.

3. Preparation of plant saponins

Ginseng saponins and bellflower saponins were isolated according to the method used by Namba *et al.*[9] Washing and slicing finely several 1.5 kg plant root were done to grind the slices in a blender and then to extract the ground with 3 liters of 99% methanol at room temperature by means of standing overnight. The mixture of aqueous methanol and ground slices was filtered through a filter paper under reduced pressure. The methanolic filtrate was concentrated under reduced pressure in a rotatory evaporator until dry. This dry extract was dispersed in 300 m*l* water to wash the dispersive liquid 4 times with 200 m*l* ether every time to eliminate fat-soluble components. To the ether-washed aqueous dispersion 200 m*l* 1-butanol saturated with water was added for shaking and separation into two phases. This partition was conducted 5 times with the 200 ml aqueous butanol each time. The combination of butanol layers obtained was concentrated to 400 m*l* and washed 3 times with 300 m*l* water each time to remove water-soluble components such as sugars. The water-washed butanol solution was concentrated to obtain the mixture powder consisted of saponins. This saponin powder was stored at room temperature inside a desiccator.

4. Extraction of cytochrome c from bovine heart mitochondria using surfactants including plant saponins

This extraction handling was designed after the methods[10] of four groups. Mitochondria prepared were dispersed in 0.01 M Tris-HCl, pH 7.8, and 0.25 M sucrose to which 0.015 M KCl was added. This dispersion was stirred for 10 min at 0℃ under the mitochondrial protein concentration of 13.6~24.6 mg/m*l*. Following this stirring the salt-treated mitochondrial dispersion was spun at 30000×g for 10 min to obtain mitochondrial pellet. This mitochondrial preparation was redispersed in the buffer solution mentioned above to be treated with surfactants and/or more concentrated KCl as follows. (1) 0.15 M KCl, (2) 0.15 M KCl and digitonin, (3) 0.15 M KCl and DMS, (4) 0.15 M KCl and ginseng saponin, (5) 0.15 M KCl and bellflower saponin. Surfactant concentration in this treatment was 0.15 mg/mg mitochondrial protein. The treatment described above was performed at 0℃ for 20 min with stirring followed by the centrifugation at 30000×g for 10 min and then by the cytochrome c analysis for the supernatant.

5. Assay of extracted cytochrome c

A spectrophotometric method was applied for analyzing the cytochrome c extracted from bovine heart mitochondria. We examined the absorbance difference ranging from 400 to 600 nm between the absorbance for the centrifugation-given supernatant and that for the supernatant reduced by sodium dithionite. The shapes of the spectra given by the original supernatant and the sodium dithionite-treated supernatant, the molar extinction coefficient of 18.5 $mM^{-1}\cdot cm^{-1}$ at 550 nm, and the absorbance

difference between the original cytochrome c and reduced cytochrome c enabled the qualification and quantification of the cytochrome.[10(b)]

6. Protein determination

The biuret method introduced by Rendina[11] and Layne[12] was used for protein quantification using a calibration curve with bovine serum albumin as a standard.

Results and Discussion

Endogenous cytochrome c-deficient mitochondria had to be prepared for the studies on confirmation of the functions of electron transfer and cytochrome c as electron carrier. Extraction of endogenous cytochrome c from mitochondrial membrane was done through the exposure to hypotonic salt medium for isolated mitochondria. Jacobs and Sanadi[10(a)] studied, in accordance with this method, on the quantitative relationship between added cytochrome c and restoration of succinate, glutamate, α-ketoglutarate, citrate, β-hydroxybutyrate, and NADH oxidations by using rat liver mitochondria which were exposed to 0.015 M KCl and then washed with 0.15 M KCl. Matlib and O'Brien[10(d)] reported, however, that endogenous cytochrome c bound to the outside of inner membrane was not extracted on suspending mitochondria in the 0.015 M KCl alone, but extracted through the disruption of outer membrane by the treatment of sufficient digitonin (0.15 mg/mg·protein) in the identical salt concentration. The impermeability to outer membrane of cytochrome c was recognized by them through this observation. Examining intact mitochondria, on the other hand, Lenaz *et al.*[10(c)] supposed the permeability to the outer mitochondrial membrane of cytochrome c. Different results have been reported on the membrane permeability and the degree of cytochrome c extraction in mitochondrion as described above. The degrees of cytochrome c extraction in the bovine heart mitochondria employing salt and surfactants such as digitonin and DMS including plant saponins were examined to ascertain whether the ginseng and bellflower saponins could be used instead of the generally-employed surfactants of digitonin and DMS in this paper.

Table 1. The cytochrome c extraction from bovine heart mitochondria using salt and salt-surfactants including salt-saponins

Treatment	Total extracted cytochrome c[a] (nmol)	Total mitochondria protein (mg)	Degree of extraction (nmol/mg·protein)
KCl	96.88	259	0.37
Digitonin	209.1	272	0.77
DMS	1284.29	316	4.06
Ginseng saponin	575.60	316	1.82

[a]Calculated from final extracted volumes and cytochrome c concentrations in them. A molar extinction coefficient of 18.5 mM^{-1}·cm^{-1} for absorbance difference at 550 nm between the spectra for the oxidized and dithionite-reduced forms is used in this calculation.

To extract cytochrome c existing in membrane, mitochondria were suspended in the hypotonic KCl and then isotonic KCl with stirring. The degree of extracted cytochrome c was 0.37 nmole/mg protein by this chloride handling (Table 1). This result obtained using a modified method after that reported by MacLennan *et al.*[10(b)] was almost identical to their value of 0.385 nmole/mg protein. The mitochondria swollen in hypotonic KCl were treated with 0.15 M KCl and surfactant digitonin of 0.15 mg digitonin/mg protein to obtain the supernatant from spinning for the determination of cytochrome c extracted. The cytochrome level extracted from this treatment was 0.77 nmole/mg protein (Table 1) which was higher than that from isotonic-chloride treatment only. This observation agrees with the fact that cytochrome c cannot be released from the suspension of isolated intact rat liver mitochondria in 0.15 M KCl alone, but can be released with the aid of digitonin which disrupts outer membrane. The level of extracted cytochrome c which was released from mitochondria by the DMS treatment whose method was identical with above-mentioned digitonin extraction using identical concentration of the surfactant was 4.06 nmole/mg protein. The cytochrome c-extraction efficiency by this DMS treatment was superior to that by digitonin. Employing both salt and surfactant for the extraction and puri-

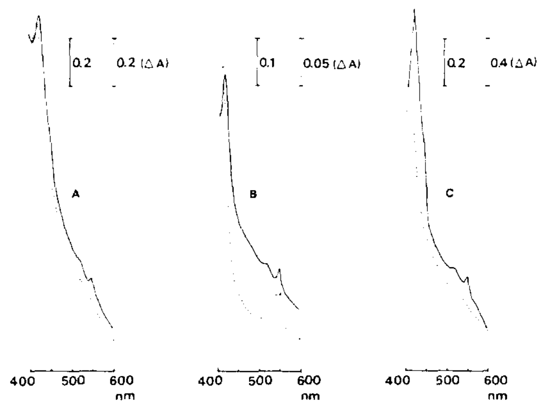

Fig. 1. Electronic absorption spectra of extracted cytochrome c solutions obtained from mitochondria treated with hypotonic salt (A), salt plus digitonin (B) and salt plus DMS (C). The dotted and solid lines represent the oxidized and reduced forms of extracted cytochrome c, respectively. All the spectra for the reduced states are alike considerably to an absolute spectrum of reduced cytochrome c in respect that they display the absorptive peak at 550 nm.

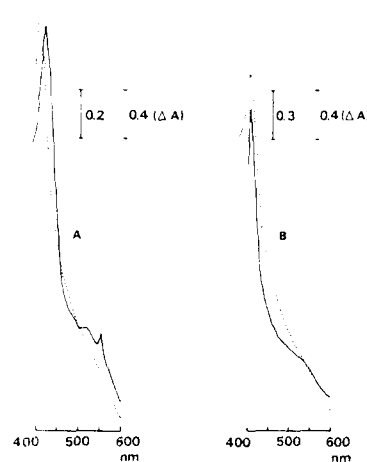

Fig. 2. Electronic absorption spectra of cytochrome c solutions extracted from mitochondria treated with the ginseng (A) and bellflower (B) saponins. The reduced spectrum (solid line) in A is similar to the standard reduced spectrum form of cytochrome c. An absolute absorptive peak at 550 nm is not shown, however, in the reduced spectrum (solid line) in B.

fication of mitochondrial-intermembrane cytochrome c must be useful. With regard to the insolubility of digitonin in water, the usage of DMS instead of digitonin appears to be more desirable. Matlib and O'Brien[10d)] described that perfect disruption of outer membrane employing digitonin was accomplished with 0.15 mg digitonin/mg protein. We added ginseng and bellflower saponins to the salt-treated mitochondria in the identical manner used for digitonin treatment and then examined cytochrome c release. Cytochrome c extraction could be performed to the extent of 1.82 nmole/mg protein with ginseng saponin treatment. This value demonstrated that the effect of ginseng saponin was superior to that of digitonin but inferior to that of DMS in the cytochrome c extraction. Concerning the solubilization of integral membrane protein such as mitochondrial cytochrome b_5, on the other hand, Lee *et al.*[13)] could not ascertain any effective outcome using ginseng saponin. This examination not for cytochrome b_5 gave rise to the idea of substitutional property of ginseng saponin for the generally-employed surfactants in the extraction of mitochondrial intermembrane cytochrome c. We examined the absorption spectra shown by the cytochrome c solutions released after the treatment with the chloride salt and surfactants including ginseng saponin. The cytochrome c was reduced with a reductant of dithionite in this checking. All the spectra examined showed an absolute peak at 550 nm which appears without fail in the reduced cytochrome c spectrum only.

For the absorption spectrum of cytochrome c solution which was prepared treating with bellflower saponin and then reduced by dithionite, the absolute reduced spectrum form of the cytochrome c was not observed (Fig. 2). The substitution for the solubilizing surfactants by bellflower saponin could not presumably be anticipated while ginseng saponins were the desirable substitute.

요　약

생체막단백질 분리 및 정제시 사용되는 전형적인 계면활성제들의 대용으로 계면활성을 지닌 식물사포닌의 사용가능성을 인삼 및 도라지사포닌에 의한 소심장 미토콘드리아 막단백질인 cytochrome c 추출효과로써 검토하였다. 이 추출효과는 막단백질 분리에

일반적으로 사용되는 고가의 계면활성제인 digitonin과 n-dodecyl-β-D-maltoside(DMS)에 의한 추출효과와 비교하였다.

Digitonin과 DMS에 의한 cytochrome c 추출은 각각 0.77, 4.06 nmole/mg·protein 정도이고 인삼사포닌은 1.82 nmole/mg·protein 정도의 추출효과가 확인되었다. 즉, 인삼사포닌은 막단백질 cytochrome c 추출에서 DMS의 효과에는 미치지 못하나 digitonin보다는 더 좋은 추출결과를 보여주고 있다. 따라서 본 연구는 막단백질의 분리정제시 사용되어온 계면활성제 대용으로 인삼사포닌의 사용 가능성을 충분히 보여주고 있다. 그러나 도라지사포닌에 의한 cytochrome c 추출효과는 관찰되지 않았다.

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