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Fiber Type Specific Presence of Glutathione Transferase Isoenzymes in the Rat Skeletal Muscle Tissue†

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=Abstract=**Glutathione transferases (GST) are a group of enzymes, majorly responsible for biochemical detoxification by conjugating glutathione to a set of hydrophobic ligands. The tissue isoenzyme pattern of the enzymes has been well illustrated with their significance of histological localization. But in the cases of muscle tissue, the GST isoenzyme pattern has not yet been clearly studied. In the present experiment, we have carried out an immunohistochemical analysis on the distribution of GST isoenzymes using anti GST-P and anti GST-L antibodies on cardiac, smooth and skeletal muscle tissues. The results showed that the intestinal smooth muscle and cardiac muscles are very weakly immunostained for both anti GST-L and P antibodies. In contrast, major bundles of skeletal muscles were positively responsive to GST-L antibody. Therefore, we compared the expression of GST-L in the muscle tissues of the soleus and plantaris, which were composed dominantly of type I fiber and type II fiber, respectively. The data indicated that only type II fibers in the plantaris muscle tissue were positive to GST-L antibody, which was confirmed by specific ATPase staining. And the soleus muscle, consisting mainly of type I fibers, contains a higher amount of GST-P isoenzyme than the plantaris muscle. Therefore, it can be suggested that the expression of GST isoenzyme can be used as a type-specific marker for the type II fiber of skeletal muscles. And moreover, the differential pattern of GST isoenzymes in those muscle tissues according to fiber types may contribute to explaining the differences in fatigue-sensitivity of muscles to exercise.**

Key Words: *Glutathione transferase isoenzymes, Type II fiber of skeletal muscle, Smooth muscle, Cardiac muscle*

INTRODUCTION

Glutathione (GSH) is the major intracellular thiol compound responsible for detoxification of a group of hazardous compounds via conjugation reaction by glutathione transferases (GST). Therefore, thve enzyme has paramount importance in cellular survival in the environment and is known to be readily induced by a variety of extrinsic and intrinsic stimuli. A complex of GST isoenzymes have been isolated and cloned in a variety of tissues and many different species (Jakoby 1978). However, in the immunological aspect, the GST isoenzymes can be classified simply into three different types such as alpha, mu and pi(Mannervik *et al.* 1985). The isoenzymes of GST are different in their substrate affinity and histological distribution. Among GST isoenzymes, the Pi form has an additional function as selenium-independent peroxidase, because of which the isoenzyme might be more concerned in oxidative stress condition as part of the defense apparatus (Park *et al.* 1990, Oberley *et al.* 1991).

Since glutathione utilization is deeply involved in scavenging the radicals and protecting the host cells, the significance of GST enzyme in some special muscle tissue such as heart tissues has been recently emphasized. In the case of human heart tissue, six different forms of GST isoenzymes are detected, among which the major form belongs to the pi class and the other five forms to the mu class isoenzyme (Tsuchida *et al.* 1990, Caccuri *et al.* 1988). Those enzymes in the aorta or heart tissues were suggested to be involved in vasodilatation by nitroglycerin and in protection from oxidative stress (Mezzetti *et al.* 1990, Tsuchida *et al.* 1990). Concerning the role of GST, it might be interesting to analyze GST in the other muscle tissues such as smooth muscle or skeletal muscle. Since all the muscle tissues are involved in contractile movement with consumption of energy and oxygen and with consequent generation of radicals, it would be pertinent to assume that the muscle tissues regardless of type would be equipped with the strong radical scavenging system including glutathione transferases. However, the presence of GST isoenzymes in those skeletal muscle tissues has not been clearly shown yet. Therefore, in the present experiment, we tried to analyze and compare the GST isoenzyme pattern in the muscle tissues of the heart, aorta, stomach, intestine and skeletal muscles from gastrocnemius, soleus and plantaris by using isoenzyme specific antibodies. And actually in our study, we have observed the muscle fiber-specific presence of the GST isoenzyme pattern; that is, type I fiber is enriched with GST-P isoenzyme, while type II fiber is mainly enriched with GST-L isoenzyme.

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MATERIALS and METHODS

Reagents

Reduced glutathione, glycine, tris(hydroxymethyl)aminomethane, nitroblue tetrazolium (NBT), 5 bromo-4 chloro 3-indolylphosphate (BCIP), ammonium persulfate and N, N, N', N'-tetramethylene diamine (TEMED) were purchased from Sigma Chemcials (St. Louis, Mo, USA). Prestained molecular weight markers, acrylamide, N,N'-methylene bisacrylamide were obtained from Bethesda Research Lab. Inc. (Gaithersburg, Md. U.S.A.), and anti-rabbit IgG-conjugated alkaline phosphatase, or protein G-conjugated peroxidase from Boehringer Mannheim Biochem. The monospecific anti GST-P antibody and anti GST-L antibody were previously prepared in our laboratory (Kwak & Park 1988, Park *et al.* 1990, Oberley *et al.* 1991). The other chemicals of analytical grade were obtained from the locally available commercial sources.

Sample preparation

The muscle tissue of the heart, aorta, stomach, small intestine and extremities were obtained from Sprague Dawley male adult rats weighing about 300g immediately after cervical dislocation. The sample tissues were washed twice with physiological saline and were subjected to mincing and homogenization. After centrifugation of the homogenates at 105,000 $\times$g for one hour, the supernatants were used as the cytosol fraction in the analysis of enzyme activities and isoenzyme pattern. And the other parts of the muscle tissues were subjected to liquid nitrogen fixation for active staining and also to formalin fixation in 10% buffered formalin for immunohistochemical analysis.

Western blot analysis

The fifty microgram cytoplasmic protein prepared from each muscle tissue was subjected to SDS-polyacrlamide gel electrophoresis. After electrophoresis, the protein fractions on the gels were electrotransfered to nitrocellulose filter papers, which were treated successively with the respective primary antibody raised against purified GST-P or GST-L (1:1000 dilution) and secondary antibody of protein G-conjugated peroxidase (1:2000 dilution) or anti rabbit IgG-conjugated alkaline phosphatase. The enzyme bands were visualized by peroxidase reactions or alkaline phosphatase reactions of the secondary antibody.

Immunohistochemical analysis

The rat muscle tissues were fixed in Carnoy's solution and embedded in paraffin. The 4μm sections were treated sequentially with the respective primary antibodies(1:500 dilution), biotinylated secondary antibody and avidin-biotin peroxidase complex followed by visualization with diaminobenzidine peroxidation after the standard routine procedure.

ATPase staining

Serial transverse sections were cut with a microtome at −20°C and stained for myofibrillar ATPase after preincubation in alkaline condition (pH 9.3) for muscle fiber type classification into slow twitch fiber and fast twitch fiber (Padykula & Herman, 1955). The 10μm frozen section of plantaris and soleus muscles were treated successively with immersion in calcum veronal buffer (pH 9.3) for 15 minutes, incubation in ATP substrate solution (0.05 M, pH 9.3) for 30 minutes at 37°C, washing with 1% calcium chloride solution and 2% cobalt chloride solution, respectively, and finaly visualization with 1% ammonium sulfide for 3 minutes. The immunohistochemistry against GST-L was performed using adjacent serial sections for comparison.

RESULTS

Western blot analysis and Immunohistochemical pattern of GST isoenzymes in the muscle tissues

The cardiac muscle of the rat responded weakly to both the anti GST-P but strongly to anti GST-L antibodies. This result indicated the relatively high presence of GST-L isoenzymes in the heart tissue. In the small intestine of the rat, low presence of GST-P was observed in the zones of intestinal villi and terminally differentiated epithelial cells. In contrast, high presence of GST-L isoenzymes was noted in those epithelial cells. However, in the smooth muscle of the small intestine, the presence of either GST-P or GST-L was low(Fig. 1).

However, it was observed that some fiber bundles of the skeletal muscle in the subcutaneous zone were strongly positive in reaction with anti GST-L antibody, which indicated the fiber-specific localization of GST-L isoenzymes(Fig. 2). And the muscles of the stomach and aorta were enriched rather with GST-L isoenzymes than with GST-P isoenzyme. The control study with liver cytosol indicated the specific presence of GST-L and the absence of GST-P in normal liver (Fig. 1).

Fiber type specificity of GST isoenzyme

The muscles of the soleus and plantaris

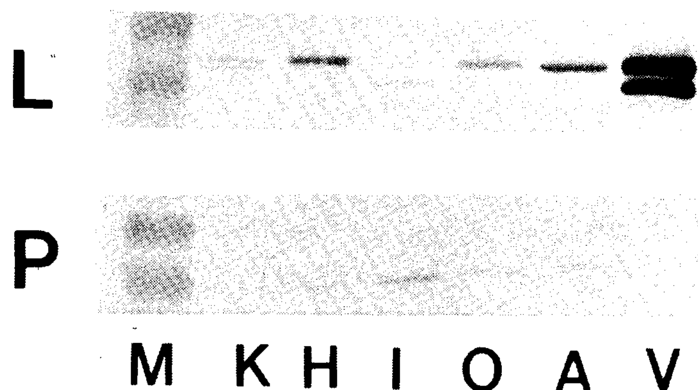

Fig. 1. Western blot analysis of GST isoenzyme pattern in the various rat muscles with the type-specific antibodies.
Upper (L) column is immunochemically stained with anti GST-L antibody, while lower (P) column, with anti-GST-P antibody. M, denotes molecular weight markers; K, skeletal gastrocnemius; H, heart; I, intestine; O, stomach; A, aorta; V, liver

were compared for the distribution pattern of GST-L as shown in Fig. 3. The soleus muscle was very weakly positive to anti GST-L antibody, while the plantaris muscle showed the mosaic pattern of GST-L positive muscle fibers. To identify the musle fiber specificity of the GST-L positive bundles in the plantaris muscle, the active myosin ATPase staining was performed on the serial frozen sections of plantaris muscle, simultaneously with GST-L antibody staining. As shown in Fig. 5, the ATPase (pH 9.3)-positive fibers were also positive to GST-L antibody reaction, which indicated that the GST-L positive muscle bundles were composed essentially of type II fibers. And the western blot analysis of the soleus and plantaris muscle with anti GST antibodies also confirmed that soleus muscle is equipped dominanly with GST-P isoenzyme, while the plantaris has the GST-L isoenzyme(Fig 4).

DISCUSSION

In the present experiment, we found that skeletal muscle tissues are different in their GST isoenzyme pattern according to muscle fiber

Fig. 2. Immunohistochemical analysis of the subcutaneous tissue of the rat with antiGST-L antibody.

types. Since the activity of glutathione transferase is related to the radical scavenging and elimination of hazardous materials, the localization of the enzyme may suggest the active involvement of such processes in certain tissues (Jakoby 1978, Chasseaud 1979). Moreover, the type-specific localization of the isoenzymes of GST would distinguish the special functions in relation with their differences in substrate affinity. In our previous studies, we have prepared the type-specific antibody to acidic form of GST, named anti GST-P antibody (Kwak & Park 1988) and antibody to alpha and mu forms of GST, collectively, named anti GST-L antibody (Park *et al.* . 1990).

The murine liver tissues are enriched with basic isoforms of GST-isoenzymes (which are named as GST-L, composed in major part by

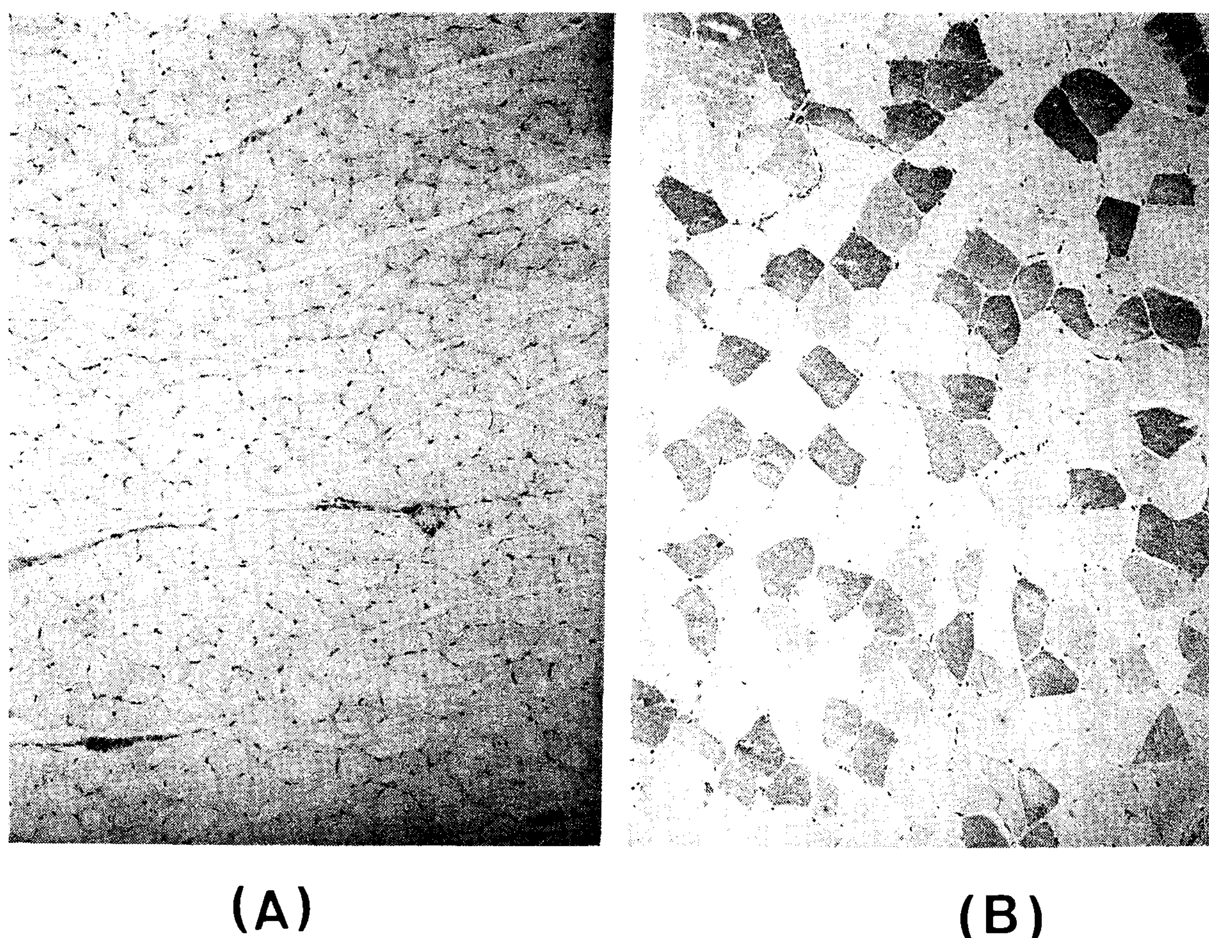

Fig. 3. Immunohistochemical analysis of rat muscles with anti GST-L antibody.
(a) soleus, (b) plantaris

alpha form isoenzymes). But if the hepatic tissue were transformed by a variety of causes, the expression of GST isoenzyme genes might shift to that of GST-P, acidic isoform. Therefore, the expression of GST-P form in the murine liver tissue can be used as the biospecific marker for transformed foci(Sato et al. 1984, Satoh *et al.*. 1985, Shea *et al.*. 1988, Park *et al.*. 1990). The substitution of the specific isoform of GST enzymes would explain the characteristics of the transformed foci in relation to cellular defense and adaptation. And the tissue distribution of GST-L and GST-P has been studied immunohistochemically and biochemically in different species which illustrated considerable differences between species (Oberley *et al.* 1991, Baars *et al.* 1987, Tateoka et al. 1987, Kwak & Park 1990, Park *et al.* 1991, Blacker *et al.*. 1991).

In the muscle tissues, the specific isoforms of GST have been isolated with low activity and small amounts, which have been named the mu class of GST isoenzymes (Suzuki *et al.*. 1987, Vorachek *et al.* 1991). However, no study on GST enzymes in the skeletal muscle tissues has been thoroughly carried out, so far. Since muscle tissues should consume ATP energy and oxygen to continue contractile movement, the generation of oxygen radicals and other waste products would be inevitable. Therefore, the distribution of GST isoenzymes in the muscle tissues of the heart, intestine and extremities would explain in part the differential nature of the muscle fibers in metabolism and mechanical efficiency.

In the present study we found that the cardiac and smooth muscle contained a very low level of enzymatic activities and a rather negligible amount of GST-L or GST-P enzymes(Fig. 1). Even though some reported results have revealed that the Mu class of GST isoenzyme is working in the cardiac and smooth muscle tissues, the amount of the isoenzyme was very low. In contrast, only certain bundles of muscle

fibers in the skeletal muscle reacted strongly with anti-GST-L antibody(Fig. 2). Since the skeletal muscle tissues are composed of different fibers, we tried to identify the type specificity of GST-L expression. For that purpose, we chose the muscle tissues of the soleus and plantaris, because the latter is composed mainly of type II fibers, while the former is composed of type I fibers(Bone 1966, Needham 1926). The immunohistochemical analysis with anti GST-L antibody revealed that only the plantaris muscle reacted positively with the antibody, which means that the aerobic soleus muscle of type I fiber is devoid of GST-L enzyme, while the anaerobic plantaris muscle of type II fiber has some amount of GST-L isoform (Fig. 3). The glutathione transferase isoenzyme pattern of the soleus and plantaris muscles was also confirmed by western blot analysis with the respective specific antibody (Fig. 4). The western blot analysis showed that soleus muscle is enriched with GST-P isoenzyme, while plantaris muscle is

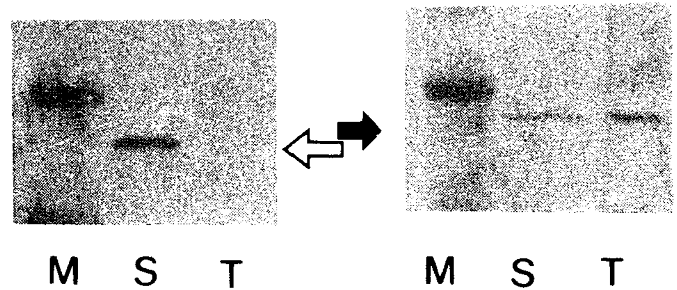

Fig. 4. Western blot analysis of skeletal muscles with type-specific anti GST antibodies.
S denotes soleus muscle while T, plantaris muscle and M, molecular weight markers. Left column (P) is stained immunochemically with anti GST-P antibody and right column (L) with anti GST-L antibody.

enriched with GST-L isoenzyme. Nonetheless, only some of the muscle bundles in the plantaris were positive in GST-L antibody reaction. To identify the type of the GST-L positive fiber in the plantaris, we performed the ATPase active stain-

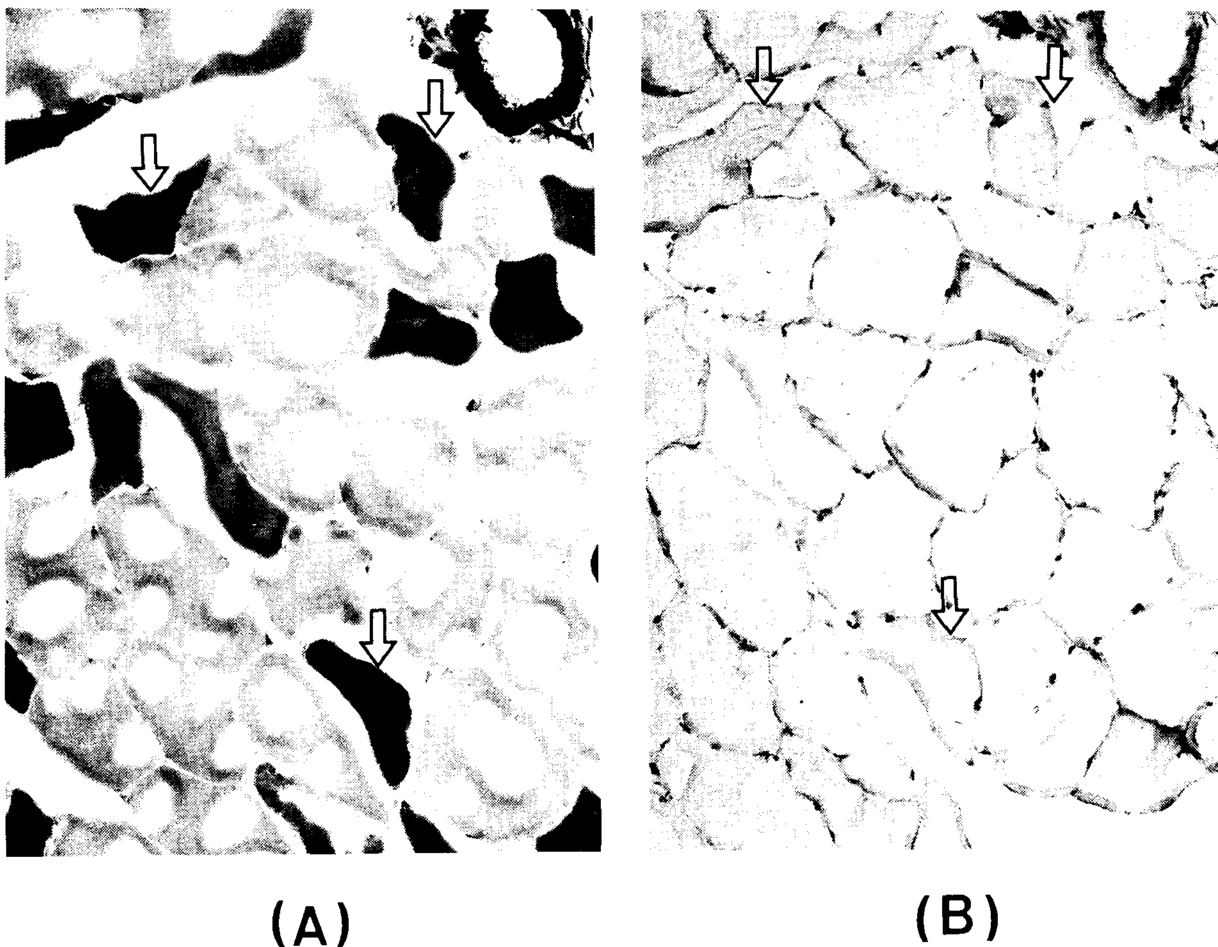

Fig. 5. Comparison of myosin ATPase activity and GST-L in the plantaris muscle.
(a) active ATPase staining; (b) anti GST-L antibody staining

ing (Fig. 5). The simultaneous analysis of plantaris muscle fibers with ATPase staining at pH 9.3 and anti GST-L antibody, showed that the GST-L positive fiber bundles were also positive in myosin ATPase staining(Saltin *et al.* 1977). These results indicated the GST-L positive fibers are actually type II fibers.

So far, the classification of muscle fiber types was dependent on active staining of the enzymes such as ATPase, succinate dehydrogenase, or phosphofructokinase etc, which limited the application of fiber type classification, because of difficulty in maintenance of enzyme activity and the likelihood of error in active staining. Therefore, the immunological positivity to GST-L antibody of the type II fiber would facilitate the specific fiber type-related research into skeletal muscles, since the antibody can work on the paraffin bloc samples without requiring the frozen sample. In addition, such a specific localization of GST-L isoform in the type II fibers of the skeletal muscle raised the question on the specific biological significance of the specific presence of the isoenzyme in the specific fiber. The plausible explanation at present is that the enriched GST-L presence in the type II fibers might be related to oxygen utilization. Type I fibers usually contain many mitochondria and an active oxygen utilization system for aerobic respiration and are responsible for slow twitch movement, while type II fibers are equipped with less mitochondria and are usually dependent on anaerobic respiration and are responsible for fast twitch movement. The higher presence of GST-P in soleus muscle of type I fibers indicated that GST-P might function as an efficient defense apparatus against oxidative stress, generated inevitably during exercise in the process of oxygen consumption.

However in the muscles of type II fiber, the oxidative stress might be less than in type I fibers, because of scarce mitochondria and less chance of oxygen radical generation. Therefore, it would be logical to assume that type II fibers contain GST-L isozyme, while type I fibers are enriched with relatively high amounts of GST-P isozyme, which has an additional selenium independent peroxidase activity. And this property would be a good clue to explain the relative fatigue-resistency of type II fibers, because richness of GST-L in those fibers might play an additional detoxifying role in scavenging the fatigue-related metabolites, generated during exercise.

In conclusion, we have observed that the isoenzyme pattern of GST was contrasting in the skeletal muscles in type specific manner; that is, type I fiber is enriched with GST-P, while type II fiber is enriched with GST-L. Therefore, presence of GST-L would be a good marker specific to type II muscle fiber.

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