Isolation and Identification of Precursor of 4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone from Isolated Soybean Protein and Shoyu

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4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone (HEMF) has been presumed to be biosynthesized by many kinds of yeast through intermediates, like D-ribulose 5-phosphate, found in the pentose−phosphate cycle. In this study, it was proved that D-xylulose 5-phosphate, one of the intermediates in the pentose−phosphate cycle, was present in both the enzymatic hydrolysates of isolated soybean protein and in shoyu "moromi mash" before the growth of yeasts in shoyu fermentation. It is also reported that D-xylulose 5-phosphate is the precursor for HEMF.

Keywords: *4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone; D-xylulose 5-phosphate; isolated soybean protein; shoyu flavor*

INTRODUCTION

4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone (HEMF) was isolated for the first time from the natural product in 1976 (Nunomura et al., 1976). It has an intense, sweet aroma and a shoyu-like flavor. HEMF is contained in a high concentration (>100 ppm) in shoyu and has the lowest odor threshold in water (<0.04 ppb; Ohloff, 1978) among the compounds found in shoyu. The odor value of HEMF is calculated to be greater than five million. Moreover, it exists only in shoyu and miso (Sugawara, 1991) and does not exist in other foods. In miso, the HEMF content is much lower than in shoyu. Accordingly, HEMF is a significant contributor to shoyu flavor and is considered to be a character impact compound of shoyu. Recently, Nagahara et al. (1992) reported that HEMF posseses antitumor activity.

HEMF is biosynthesized through the pentose−phosphate cycle by shoyu yeasts. Not only shoyu yeasts but also the other yeasts employed for alcoholic beverages and single-cell protein can change intermediates, such as D-ribulose 5-phosphate, to HEMF (Sasaki et al., 1991).

This report describes the purification and identification of a precursor of HEMF from shoyu and isolated soybean protein.

MATERIALS AND METHODS

Chemical Reagents. D-Glucose 6-phosphate monosodium salt, D-ribulose 5-phosphate barium salt, D-xylulose 5-phosphate sodium salt, D-ribose 5-phosphate barium salt, and D-sedoheptulose 7-phosphate barium salt were purchased from Sigma Chemical Company (St. Louis, MO). All other reagents were of reagent grade or the best grade available commercially.

Dialysis Membrane. Two types of dialysis membrane from Spectrum Medical Industries Inc. (Los Angeles, CA) were used. One membrane had a cutoff molecular weight (MW) of 1000 and a flat width of 45 mm, and the other had a cutoff MW of 3500 and flat width of 54 mm.

Plant Material. All of the plant material mentioned later were milled, and the particles that passed a 200-mesh sieve were collected.

Defatted Soybeans. Defatted soybeans were denatured without water but with superheated steam at a gauge pressure of 4−8 kg/cm^2 at 200−287 °C for not less than 15 s so that the soybean protein became susceptible to enzyme action.

Isolated Soybean Protein. A commercial soybean protein product was used (FUJI PRO 620; (Fuji Purina Protein Company, Ltd., Tokyo, Japan).

Wheat. Wheat was cooked without water in a manner similar to that used for the soybeans.

Wheat Gluten. A commercial wheat gluten product was used.

Peanut. A commercial peanut product, unhulled and roasted, was defatted with acetone.

Enzymes. *Protease.* Crude enzymes powder No. 814 (I) was prepared from the water extract of wheat bran culture of *Aspergillus sojae* according to the procedure reported by K. Hayashi et al. (1967). Molsin (M) from *Aspergillus saitoi* was supplied by Seishin Corporation (Tokyo, Japan). Thermoase (T) from *Bacillus thermoproteolyticus* Rokko was supplied by DAIWA KASEI Company, Ltd. (Osaka, Japan).

Cellulase. Meicelase (ME) from *Trichoderma koningi* was supplied by Meiji Seika Kaisha, Ltd. (Tokyo, Japan). Driserase (D) from *Irpex lacteus* was supplied by Kyowa Hakko Kogyo Company, Ltd. (Tokyo, Japan).

Hemicellulase. Hemicellulase I (TH) from *Aspergillus niger* was supplied by Tokyo Kasei Organic Chemicals (Tokyo, Japan). Hemicellulase II (H) from crude enzymes was prepared from the water extract of wheat bran culture of *Aspergillus sojae* according to the procedure reported by T. Kikuchi et al. (1971).

Microorganisms. The yeast strains used in this investigation were stock cultures from our laboratory.

Media. *Basal Medium.* One hundred grams of shoyu koji was dissolved in 900 mL of distilled water and incubated in a water bath at 58 °C for 6 h. The mixture was boiled for 15 min. The hot liquid was vacuum-filtered through filter paper and sterilized for 15 min at 15 lb over-pressure. The pH of the filtrate was 6.5.

Enzyme-Added Medium. Seven kinds of enzyme agents were added to the basal medium in a concentration of 1.6%. The sample was blended for 10 min in a Waring blender and then centrifuged at 3000 rpm for 10 min at 5 °C on a Hitachi centrifuge (model 05 PR-22) to remove insoluble parts. The supernatant was sterilized by passage through 0.22-μm membrane filter.

Medium for Shoyu Yeasts. This medium was prepared by adding 17% NaCl (w/v), 5% glucose (w/v) to the basal medium and then adjusting the pH to 4.8 with diluted lactic acid.

Cultivation. Five milliliters of enzyme-added medium and 1 g of the steam-sterilized fine powder of plant materials were dispensed into a 12-mL sterilized screw-capped vial. The mixture was incubated at 37 °C for 24 h. Soon after the incubation, sodium chloride at 17% (w/v) was added to the

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Table 1. Conditions for Purification of Precursor for HEMF

equipment	step 1	step 2	step 3
pump	model CCPM (Toso Co., Inc.)	model CCPM Prep (Toso Co., Inc.)	(1) model L-6200 (Hitachi Co., Inc.) (2) model L-6000 (Hitachi Co., Inc.)
valve	model 7125 (Rheodyne Inc.)	model 7125 (Rheodyne Inc.)	model 7125 (Rheodyne Inc.)
(loop, μL)	500	500	500
mixer	—	—	dynamic mixer, model MX 8010 (Toso Co., Inc.)
detector	RI: model Shodex SE-62 (Showa Denko Co., Inc.)	RI: model Shodex SE-62 (Showa Denko Co., Inc.)	UV, model L-4000 (Hitachi Co., Inc.)
column	Shodex KQ 802 (Showadenko Co., Inc.)	TSK gel CW-35 S (Toso Co., Inc.)	TSK gel Amide 80 (Toso Co., Inc.)
(size)	(8 mm i.d. × 300 mm)	(20 mm i.d. × 300 mm)	(4.6 mm i.d. × 250 mm)
mobile phase	distilled water (1000):25%NH_4OH (0.1)	distilled water (1000):25%NH_4OH (0.1)	(A) CH_3CN (90)−5 mM H_3PO_4 (10) (B) CH_3CN (20)−5 mM H_3PO_4 (80)
(mL/min)	1.0	1.0	1.0
mode	isocratic	isocratic	gradient: 0 min, 100% A, 0% B; 120 min, 0% A, 100% B; 130 min, 100% A, 0% B
injection size (μL)	20	20	10

mixture. The mixture was inoculated with 10 μL of cell suspension of the strain *Zygosaccharomyces rouxii* (no. 210, ATCC 13356) that represented the shoyu yeast. The vial with the inoculated mixture was loosely capped, and the mixture was incubated at 30 °C for 20 days. The vial was shaken once every 2 or 3 days to accelerate the growth of the yeasts.

Determinations of HEMF and Ethanol. Determinations of HEMF and ethanol were carried out according to the methods described in our previous paper (Sasaki et al., 1991).

Gas Chromatography (GC) Analysis of Sugar Phosphates. *Preparation of Tetramethylsilane (TMS) Derivatives of Sugar Phosphates.* Preparation of TMS derivatives of sugar phosphates was performed by the method described by Harvey et al. (1973).

Capillary GC. Analysis of TMS derivatives of sugar phosphates were carried out the same way as determination of HEMF, except for the program for GC separation. The oven temperature was held at 40 °C for the first 1 min and then programmed to rise to 200 °C at 3 °C/min and held at 200 °C for 90 min. One microliter of silylation mixture containing trimethylsilyltrifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS), and acetonitrile (2:1:2) was injected in the splitless mode with purge time of 0.8 min.

Isolation of the Precursor for HEMF. *Hydrolysis of Isolated Soybean Protein by Enzymes.* The enzymatic powder (18.9 g) containing seven enzymes (2.7 g each), was added to 135 mL of distilled water. The mixture was centrifuged at 3000 rpm and 5 °C for 10 min. One hundred and eighteen milliliters of the supernatant was sterilized by passing through a 0.22-μm filter unit. The total of the filtrate (116 mL) and 30 g of isolated soybean protein sterilized at 120 °C for 20 min were then placed in a 300-mL Erlenmeyer flask that was previously sterilized by dry heat. The mixture was incubated at 37 °C for 24 h. After incubation, the hydrolysate was centrifuged at 3000 rpm and 5 °C for 10 min. The supernatant was then filtered through an 0.22-μm filter unit. The yield of the filtrate was 94 mL.

Dialysis of the Hydrolysate of the Isolated Soybean Protein. Eighty-five milliliters (corresponding to 27.13 g of the original protein; i.e., 30 g × 85/94) of 94 mL of the hydrolysate was diluted with distilled water to 120 mL and divided into three portions (40 mL each). Each portion was put into a dialysis membrane (cutoff MW, 3500 Da; diameter, 54 mm; length, 15 cm) and exhaustively dialyzed against three changes of deionized water in the cold. The diffusate and the dialysate were each concentrated to 100 mL in a rotary evaporator. Therefore, 3.69 mL (100 mL/27.13 g) of each concentrate corresponds to 1 g of isolated soybean protein. The concentrated dialysate (100 mL) was further dialyzed in another dialysis membrane (cutoff MW, 1000) in a similar manner.

This procedure resulted in preparation of three fractions with the following ranges of MW: >3500; 1000−3500; and <1000. The dry material (3.69 mL, corresponding to 1 g of isolated soybean protein) of each fraction was obtained by evaporation to dryness on a water bath at 45 °C. To screen for the active fraction containing the precursor, the strain *Zygosaccharomyces rouxii* (no. 210, ATCC13356), representing the shoyu yeast, was cultured in a 10-mL vial containing 2 mL of the medium for shoyu yeast and the dried matter of each fraction obtained by the dialysis corresponding to 1 g of isolated soybean protein. The content of HEMF in the cultured broth was determined by the GC method.

Purification by HPLC. The HPLC apparatus and the conditions for purification steps 1, 2, and 3 are summarized in Table 1.

Step 1 (Fractionation by Shodex KQ 802 Column). In this step, 3690 μL (corresponding to 1 g of isolated soybean protein) of the 100 mL of dialysate with a MW of <1000 were divided into four fractions (Fr 1−4). Each fraction was concentrated under reduced pressure to a volume of 3000 μL; then, 750 μL of that volume, which corresponds to 250 mg of isolated soybean protein, was further evaporated to dryness in a rotary evaporator. The other 2250 μL was stored frozen at −20 °C. To screen for the active fraction containing the precursor, the strain *Zygosaccharomyces rouxii* (no. 210, ATCC13356) was cultured in a 1.5-mL vial containing 500 μL of the medium for shoyu yeast supplemented with each concentrate fraction (1−4) corresponding to 250 mg of isolated soybean protein. After that, the content of HEMF in the fermented broth was determined. Fraction 3, in which the precursor for HEMF was found, was further separated by preparative HPLC on a TSK gel CW-35 S column.

Step 2 (Fractionation by TSK Gel CW-35 S Column). Seven hundred and fifty microliters (corresponding to 250 mg of isolated soybean protein) of the 2250 μL of fraction 3 prepared by step 1 was separated into nine fractions (1−9) by repeated preparative HPLC with a TSK gel CW-35 S column. Each fraction was concentrated under reduced pressure to a volume of 1000 μL, and 500 μL (corresponding to 125 mg of isolated soybean protein) of each fraction were further concentrated to near dryness under reduced pressure. Each concentrate was dissolved in 125 μL of the medium for shoyu yeast, and the production of HEMF was checked after culturing as already described. The other 500 μL of each fraction was stored frozen at −20 °C. Fraction 1, in which the precursor for HEMF was found, was purified by step 3.

Step 3 (Purification by TSK Gel Amide 80 Column). Five hundred microliters (corresponding to 125 mg of isolated soybean protein) of fraction 1 containing the precursor of HEMF was purified again with a TSK gel CW-35 S column as already described and concentrated to dryness under reduced pressure. The pale yellow solid thus obtained was dissolved in 100 μL of distilled water. Ten microliters of the aqueous solution was applied onto a column of TSK gel Amide 80 and fractionated with the guidance of a UV detector. The peak at ∼18 min was collected. The purified fraction was concentrated under reduced pressure to a volume of ∼30 μL, and then the concentrate was directly analyzed by the secondary ion mass spectrometric (SIMS) method.

Table 2. Enzymatic Degradation of Protein Materials and HEMF Production in the Hydrolysates by *Z. rouxii* No. 210, ATCC 13356 in Stationary Culture[a]

no.	substrate	enzyme[b]	EtOH[c] (%)	HEMF[d] (ppm)
1	basal medium only	—	0.64	7.87
2	basal medium only	protease(I + M + T) + cellulase(D + ME) + hemicellulase(TH + H)	0.42	3.53
3	defatted soybean	—	2.87	3.71
4	defatted soybean	protease(I)	3.25	26.45
5	defatted soybean	protease(I + M + T) + cellulase(D + ME)	0.70	43.79
6	defatted soybean	protease(I + M + T) + cellulase(D + ME) + hemicellulase(TH + H)	3.70	51.90
7	wheat powder	protease(I + M + T) + cellulase(D + ME) + hemicellulase(TH + H)	5.25	7.02
8	wheat gluten	protease(I + M + T) + cellulase(D + ME) + hemicellulase(TH + H)	1.63	18.82
9	isolated soybean protein	protease(I + M + T) + cellulase(D + ME) + hemicellulase(TH + H)	2.12	57.78

[a] I, crude enzymes powder no. 814; D, Driserase; M, Molsin; ME, Meicelase; TH, hemicellulase I; H, hemicellulase II. [b] One gram of fine powder of defatted soybean, wheat, wheat gluten and isolated soybean protein suspended in 5 mL of enzymes added (2% each) the aqueous extract of shoyu koji was hydrolyzed at 37 °C for 24 h. The medium consisted of the following additions to the hydrolysate: NaCl, 17%; glucoase, 5%. Organism and culture: *Z. rouxii* no. 210, ATCC 13356, was cultured at 30 °C for 20 days in stationary culture. [c] Ethanol. [d] 4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone.

SIMS Analysis. The fraction isolated by HPLC with the TSK gel Amide 80 column was directly analyzed by SIMS under the following conditions. SIMS and the metastable ion spectra were recorded on a Hitachi M-80B mass spectrometer with a Hitachi M-0101 mass data system. The SIMS gun (xenon ions) was operated at 8 kV. The ion acceleration voltage was 3 kV. About a 1-μL aliquot of an aqueous sample solution was mixed with 4 μL of matrix and 4 μL of 8 % NaOH additive and then placed on the SIMS platinum target. Glycerol was used as the matrix. Metastable ion spectra were obtained by linked scan at constant *B*/*E*.

RESULTS AND DISCUSSION

Screening of Raw Material for Isolation of the Precursor of HEMF. HEMF is presumed to be biosynthesized through the pentose−phosphate cycle by shoyu yeasts. Not only shoyu yeasts, but also the other yeasts employed for alcoholic beverages and single-cell protein can change intermediates such as D-ribulose 5-phosphate to HEMF. However, the existence of HEMF was found only in shoyu and miso, not in other foods. From this information, it could be expected that the precursor of HEMF might be contained in the raw materials for shoyu. To confirm this hypothesis, the following experiments were performed. Trace amounts of HEMF have been shown to arise from the precursor in the basal medium (Sasaki et al., 1991), so it was expected that only a slight amount of HEMF would be produced in the fermented broth with the basal medium or the enzymes-added basal medium alone (Table 2, samples 1 and 2). As a next step, the yeast was inoculated into the the basal medium supplemented with defatted soybeans, which are one of the raw materials for shoyu. The result, presented in Table 2 (sample 3), also indicates that production of HEMF was small.

In light of the results just described, we degraded the defatted soybeans by protease prior to the cultivation of the yeast. The results, shown in Table 2 (sample 4), show that a fair amount of HEMF was produced compared with the enzyme-free sample (Table 2, sample 3). This result suggested that the precursor of HEMF might be eluted by enzymatic degradation of defatted soybeans.

To check the ability of the other enzymes to elute the precursor for HEMF from defatted soybeans, a total of seven enzymes belonging to protease, cellulase, and hemicellulase were selected from commercial origin. In the case of the samples hydrolyzed by the combination of proteases and cellulases, the production of HEMF was considerably high compared with the hydrolysate by protease only (Table 2, sample 5). Furthermore, degradation by a combination of all seven enzymes showed the greatest increase in production of HEMF (Table 2, sample 6). From these results, a mixture of seven enzymes was chosen for the hydrolysis of defatted soybeans.

When the defatted soybeans were replaced with wheat, which is another raw material for shoyu, the production of HEMF was absent or present in very low quantities (Table 2, sample 7). But, the addition of wheat gluten was increased the production of HEMF greatly (Table 2, sample 8). Moreover, the result in sample 9 of Table 2 indicates that the content of the precursor compared favorably with the data for defatted soybeans. These results suggest that the precursor of HEMF might be present in the protein fraction of soybeans and wheat, and the greater part of HEMF present in shoyu was derived from soybeans.

Isolated soybean protein is partially purified by extraction and precipitation, so it would be expected to facilitate the purification of the precursor of HEMF. Therefore, the isolated soybean protein was screened for the starting material to isolate the precursor for HEMF.

Dialysis of Enzymatic Hydrolysate of Isolated Soybean Protein. Hydrolyzed soybean protein (isolated soybean protein) was dialyzed with two different dialysis membranes, with cutoff MWs of 1000 and 3500 Da, and separated into three fractions with the following ranges of MW: >3500; 1000−3500; and <1000. HEMF was detected in the diffusate obtained from the membrane with the MW cutoff of 1000 (MW >3500: 5.25 ppm; MW, 1000−3500: 6.13 ppm; MW, <1000: 61.65 ppm). This result showed that the precursor for HEMF is a low-MW compound, with a MW of 1000 Da or less.

Purification of the Precursor by HPLC. The active fraction obtained by dialysis was subjected to HPLC with a Shodex KQ 802 column. A typical chromatogram is shown in Figure 1. The third fraction containing the precursor was further purified by HPLC with a column of TSK gel CW-35 S column. The results in Figure 2 show that the retention time of the first fraction containing the precursor coincided with those of D-xylulose 5-phosphate, D-glucose 6-phosphate, and

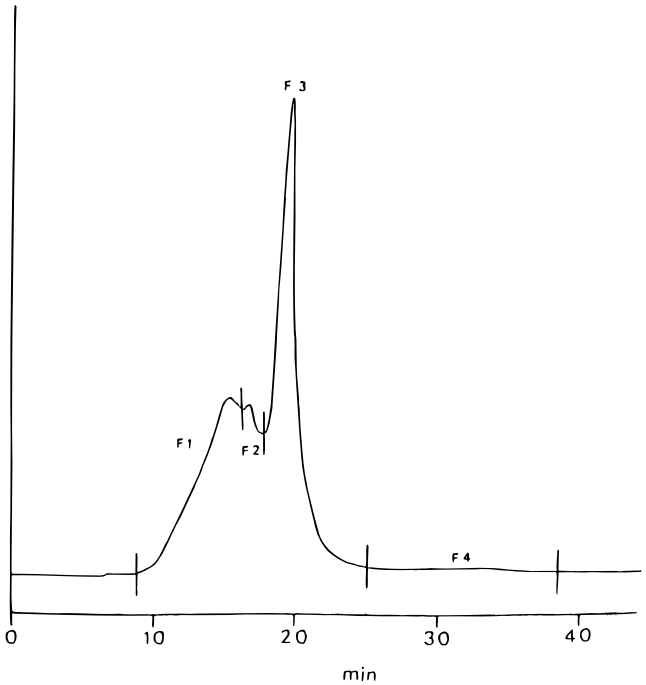

Figure 1. Typical HPLC chromatogram of the enzymatic hydrolysate of isolated soybean protein. A Shodex KQ 802 column and distilled water:25% NH_4OH (100:0.01) were used; other conditions are as in Table 1.

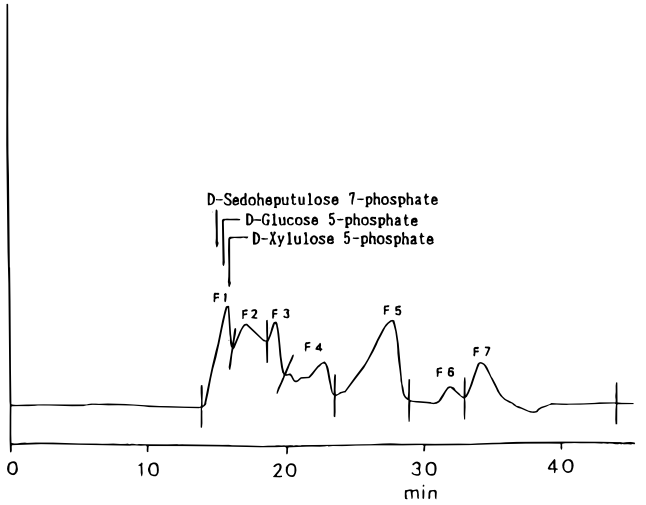

Figure 2. Representative HPLC chromatogram of fraction 3, purified by Shodex KQ 802 (D-xylulose 5-phosphate, D-glucose 6-phosphate, and D-sedoheputulose 7-phosphate (marked by arrows). A TSK gel CW-35S column and distilled water:25% NH_4OH (100:0.01) were used; other conditions are as in Table 1.

D-sedoheputulose 7-phosphate (marked by arrows). Final purification was carried out by HPLC on a TSK gel Amide 80 column. A typical chromatogram is shown in Figure 3.

The active fractions on chromatograms in Figures 2 and 3 had almost the same retention time as those of the compounds belonging to the sugar phosphate group. These results suggest that the compound belonging to the sugar phosphate group might be the precursor for HEMF.

On the other hand, it was proved by HPLC analysis that a large amount of some neutral oligosaccharides was contained in fraction 6 prepared by a TSK gel CW-35 S column. D-Threose was found for the first time in the enzymatic hydrolysate of isolated soybean protein.

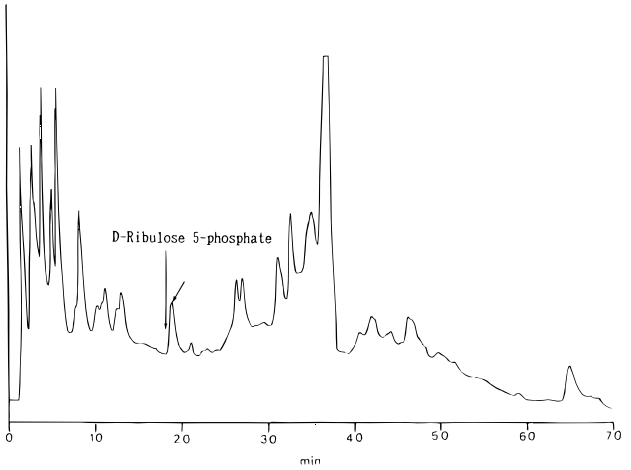

Figure 3. Representative HPLC chromatogram of fraction 1 purified by TSK gel CW-35S column (D-ribulose 5-phosphate indicated by arrow). A TSK gel Amide 80 and acetonitrile:5 mM H_3PO_4 (90−20:10−80) were used; other conditions as in Table 1.

Identification of D-Xylulose 5-Phosphate by SIMS and GC Analysis. The purified sample (arrow peak in Figure 3), collected by TSK gel Amide 80 and authentic samples (namely, D-xylulose 5-phosphate, D-ribose 5-phosphate, and D-ribulose 5-phosphate) were analyzed by SIMS, and their spectra are exhibited in Figure 4 (a−d, respectively). The results in Figure 4 indicate that the spectrum of the isolated sample and that of the authentic D-xylulose 5-phosphate coincide perfectly with each other. Moreover, as shown in Table 3, the TMS derivative of the purified sample from isolated soybean protein had a retention time identical to that of authentic TMS derivative of D-xylulose 5-phosphate. From these results, the compound purified from isolated soybean protein was identified as D-xylulose 5-phosphate. Because HEMF has been presumed to be biosynthesized through intermediates like D-xylulose 5-phosphate found in the pentose−phosphate cycle by yeasts (Sasaki et al., 1991), it was concluded that D-xylulose 5-phosphate was the precursor for HEMF in the isolated soybean protein. D-Xylulose 5-phosphate was also found in shoyu mash before the growth of yeasts. Namely, the liquid part of the mash was directly collected by HPLC under the same conditions and with the same column (TSK gel Amide 80) as used in the separation of the fractions from isolated soybean protein. The SIMS spectrum of the purified fraction agreed exactly with that of D-xylulose 5-phosphate.

As discussed earlier, all sugar phosphates in the pentose−phosphate cycle have almost the same retention time in HPLC analysis with TSK gel Amide 80. If sugar phosphates other than D-xylulose 5-phosphate are present in isolated soybean protein or shoyu, the sugar phosphate should be detected in SIMS. However, no such compounds were found in the collected fractions. These results lead to the conclusion that D-xylulose 5-phosphate is the only precursor in soybean and wheat or in shoyu. Moreover, HEMF was also found in the fermentation broth with hydrolysates of peanuts (HEMF: 43.34 ppm), so it is presumed that the precursor in these materials was also D-xylulose 5-phosphate.

It is reasonable that D-xylulose 5-phosphate in the soybeans and wheat could be derived from an intermediate of the Calbin−Benson cycle in photosynthesis. But, its content seems to be rather too large to be a mere

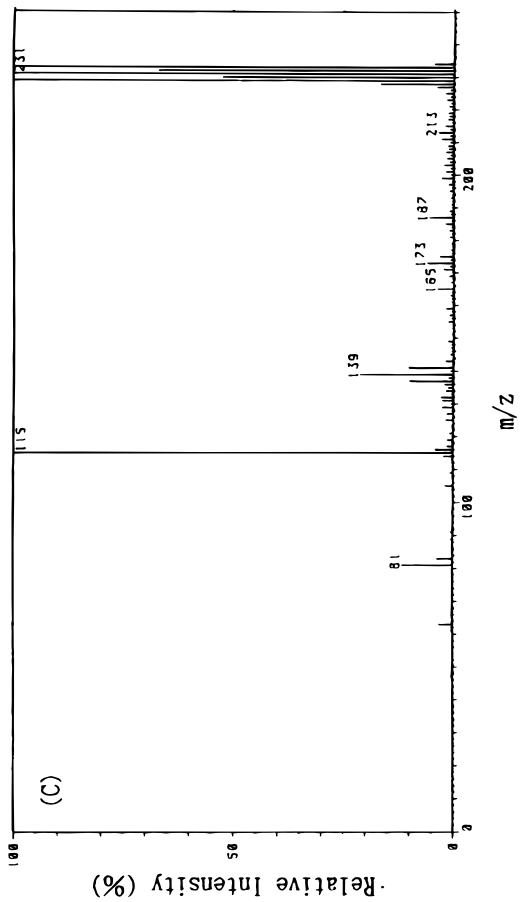

Figure 4. Low-resolution SIMS mass spectra (linked scan mode) of (a) the enzymatic hydrolysate of isolated soybean protein purified by HPLC; (b) authentic D-xylulose 5-phosphate, (c) D-ribulose 5-phosphate, and (d) D-ribose 5-phosphate.

Table 3. Retention Times (RT) of TMS Derivatives of Purified Sample from Isolated Soybean Protein Compared with Those of Authentic Sugar Phosphate[a]

sample	RT, min
D-ribose 5-phosphate	62.026
D-ribulose 5-phosphate	64.373
D-xylulose 5-phosphate	64.800
sample purified from isolated soybean protein	64.800

[a] Operating conditions were as described in the text.

intermediate in photosynthesis (in liquid part of shoyu mash before fermentation: 738.9 ppm), judging from the results of the isolation/purification process discussed in this study. However, it is necessary to determine D-xylulose 5-phosphate precisely in soybeans, wheat, and so on. Furthermore, it is very interesting that only D-xylulose 5-phosphate of many sugar phosphates among intermediates in photosynthesis exists in the plants. On the other hand, a large quantity of the many kinds of the phosphatase is known to be produced by koji mold in the koji making process. Nevertheless, the presence of a fairly large quantity of D-xylose 5-phosphate in shoyu suggests that phosphatase, which is capable of hydrolyzing D-xylulose 5-phosphate, is present in only trace quantities, if at all.

The following mechanism responsible for the formation of HEMF in shoyu was elucidated on the basis of the results of this study. Soybeans and wheat are first hydrolyzed, and D-xylulose 5-phosphate is eluted by the actions of the enzymes produced by koji mold. Then, the D-xylulose 5-phosphate is transformed into HEMF by the action of shoyu yeasts. Koji mold, with regard to biosynthesis of HEMF, plays only the role in the hydrolysis of soybeans and wheat, and the subsequent release of D-xylulose 5-phosphate.

Future studies are needed to elucidate the reason why a fair amount of D-xylulose 5-phosphate exists in plants and if D-xylulose 5-phosphate is included in polymer compounds, such as protein, or adsorbed on them.

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