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REMOVAL OF OLIGOSACCHARIDES FROM SOY MILK BY AN ENZYME FROM ASPERGILLUS SAITOI

SUMMARY—As part of a program to reduce the flatulence-inducing tendency of soy milk, a method for the enzymatic removal of galacto-oligosaccharides by means of an enzyme preparation from Aspergillus saitoi *was investigated. It was found that a partially purified preparation possessing both α-galactosidase and invertase, yet free from protease, could be obtained easily from a commercial* A. saitoi *acid-protease product by means of a simple molecular sieving procedure. The α-galactosidase exhibited its optimum pH between 5.0 and 5.5, and seemed to be stable between pH 4.0 and 8.0. The optimum temperature was found at about 55°C; however, the enzyme itself was inactivated by maintaining it at 70°C for 30 min. These properties appeared suitable for the enzymatic treatment of soy milk. p-Chloromercuribenzoate, N-bromosuccinimide, $HgCl_2$, $AgNO_3$ or $CuCl_2$ showed strong inhibitory effects on the enzyme. The presence of 1×10^{-2} M galactose caused only slight inhibition. Km value of the enzyme with melibiose as a substrate was found to be 3.11×10^{-3} M and the molecular weight of the enzyme estimated to be about 290,000 on the basis of a gel filtration technique. Investigations by means of thin-layer chromatography indicated that the addition of small amounts of this enzyme preparation to soy milk resulted in complete hydrolysis of galacto-oligosaccharides. The practicability of the present method was also discussed from an economic viewpoint.*

INTRODUCTION

SOY MILK has been given considerable attention as an economical high-protein beverage that can help overcome widespread protein deficiencies. However, there remain a number of qualitative problems which must be solved for this product to obtain a wider acceptance.

One of these problems is its tendency to induce flatulence, often accompanied by an uncomfortable feeling of fullness and intestinal activity. Recently, it has been suggested by some investigators that the flatulence caused by soy products could be due, at least in part, to their relatively high contents of galacto-oligosaccharides, especially stachyose and raffinose (Murphy, 1963; 1964 a; 1964 b; Burr, 1967; Rackis et al., 1967; Steggerda, 1967). On the basis of this hypothesis, flatulence in soy milk should be reduced by removal or decomposition of these oligosaccharides.

In preliminary studies by one of the authors (H.S.), it was found that considerable numbers of fungal strains belonging to the genus *Aspergillus* exhibited powerful abilities to produce galacto-oligosaccharide decomposing enzymes such as α-galactosidase (E.C.3.2.1.22) or invertase (E.C.3.2.1.26). Furthermore, some of the commercial enzyme products prepared from the same fungi contained considerable activities of both enzymes (manuscript in preparation).

This paper demonstrates that the oligosaccharides in soy milk can be hydrolyzed almost quantitatively by an enzyme partially purified from a commercial product. Some characteristics of the α-galactosidase which play a main part in the hydrolysis of the oligosaccharides, the qualitative changes of the oligosaccharides during the enzyme treatment, and the economical practicability of the present method are also described and discussed.

MATERIALS & METHODS

Soy milk

Freeze-dried raw soy milk prepared from Harosoy 63 variety was supplied by Dr. D. Fukushima, Kikkoman Shoyu Co., Ltd., Noda, Chiba, Japan. The chemical analysis of this material indicated the following composition: moisture 2.79%, crude protein (Kjeldahl N × 5.71) 41.90% and crude fat 24.8%.

Source of enzyme

A commercial acid-protease product, Molsin (Lot No. M19, 193) from *A. saitoi*, obtained from Seishin Pharmaceutical Co., Ltd., Tokyo, was used as a source of enzyme. 1 g of the enzyme showed 620×10^3 units of α-galactosidase activity.

Assays of enzyme activities

The reaction mixture for the assay of α-galactosidase (E.C.3.2.1.22) activity was composed as follows: 1 ml of 1% (w/v) melibiose dihydrate, 2 ml of 0.1 M acetate buffer, pH 5.0 and 1 ml of the enzyme solution. The reaction mixture was incubated at 40°C for 1 hr, then placed into a boiling water bath for 10 min. Glucose was estimated by a glucose oxidase reagent, Glucostat, according to the directions of the manufacturer, Worthington Biochemical Corp., Freehold, N.J. 1 unit of enzyme activity was defined as the amount of activity which liberated 1 mg of glucose under the conditions just mentioned. The activities of β-galactosidase (E.C.3.2.1.23), α-glucosidase

Table 1—Partial purification of enzyme preparation from Aspergillus saitoi.

	α-Galactosidase			Invertase			Protease		
	Total activity (units)	Specific activity (units/mg)	Yield (%)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Dialysate	298×10^5	3,260	100.0	114×10^6	12,500	100.0	155×10^{-2}	17.1×10^{-5}	100.0
Acetone ppt.	298×10^5	4,980	100.0	61×10^6	10,100	53.0	87×10^{-2}	14.7×10^{-5}	56.4
Ultrafiltrate	284×10^5	69,300	95.4	21×10^6	50,700	18.2	3×10^{-2}	6.6×10^{-5}	2.0
Gel filtrate	226×10^5	181,000	75.9	9×10^6	72,800	8.0	0	0	0.0

(E.C.3.2.1.20), β-glucosidase (E.C.3.2.1.21) and invertase (E.C.3.2.1.26) were assayed in the same manner using 1% (w/v) solutions of lactose monohydrate, maltose monohydrate, cellobiose and sucrose, respectively, as substrates. In both cases of α-, and β-glucosidases, 1 unit of enzyme activity corresponded to 2 mg of glucose liberated. In investigating the effects of inhibitors or activators on the α-galactosidase activity, p-nitrophenyl-α-D-galactopyranoside was employed as a substrate, since it was necessary to avoid inhibitor or activator effects on glucose oxidase. In this case, the reaction mixture consisted of 1 ml of 0.5% (w/v) p-nitrophenyl-α-D-galactopyranoside in 0.1 M acetate buffer, pH 5.0, 1 ml of inhibitor or activator and 1 ml of the enzyme solution. The reaction was carried out at 40°C for 15 min and stopped by addition of 1 ml of 10% (w/v) trichloroacetic acid. After addition of 9 ml of 0.3 N NaOH to the reaction mixture, the increase of the optical density of the mixture was estimated at 420 mμ. Protease activity was assayed at pH 6.2 according to the improved Hagihara's method B (1954). In this method, 1 unit of protease corresponded to 1 meq of tyrosine liberated from milk casein per min at 30°C.

Estimation of enzyme protein

Lowry's method (Lowry et al., 1951) was employed for the estimation of protein in the enzyme preparation and bovine hemoglobin (2× cryst.) was used as a standard protein.

Purification procedures of enzyme

The partially purified α-galactosidase, which had some other glycosidases such as invertase, although it was free from protease, was prepared from Molsin by the following procedures: All operations were carried out at 2°C. 50 g of Molsin were dissolved in 250 ml of water and dialyzed overnight with a fishskin tubing against running tap-water. After removal of insoluble material by centrifugation, the enzyme solution was brought up to a volume of 500 ml with distilled water. The same volume of cold acetone was then poured into the resultant solution, the precipitate formed collected, washed twice with 50% (v/v) aqueous acetone and pure acetone successively, then dried in vacuo. After the acetone precipitation, recovery of α-galactosidase activity was almost quantitative. The acetone powder was dissolved in 1,000 ml of 0.05 M acetate buffer, pH 5.5, and the solution concentrated to a volume of 100 ml by use of an Amicon ultrafiltration cell (Amicon Corp., Cambridge, Mass.) A Diaflo XM-100 membrane was employed under an air pressure of 1.5 kg per sq cm.

After addition of another 1,000 ml of 0.05 M acetate buffer, pH 5.5, the solution was concentrated again to a final volume of 20 ml. A 10-ml aliquot of the above enzyme solution was applied to a 2.0- by 140-cm column of Sephadex G-200 equilibrated with 0.05 M acetate buffer containing 0.1 M KCl, pH 5.5. The column was eluted with the same buffer at a flow rate of 15 ml per hr and 10-ml fractions collected.

Figure 1 shows the elution profile of α-galactosidase from the column. The fractions showing intense α-galactosidase activity were pooled, concentrated and desalted by means of a collodion bag assembly. The concentrate was then freeze dried and employed in subsequent experiments as a partially purified enzyme

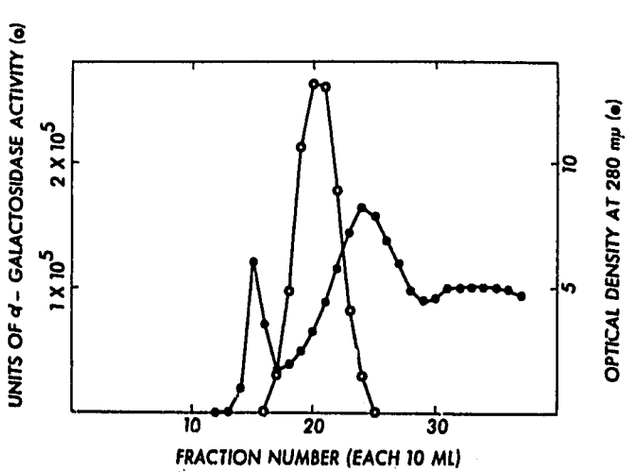

Fig. 1—Gel filtration of α-galactosidase on a Sephadex G-200 column.

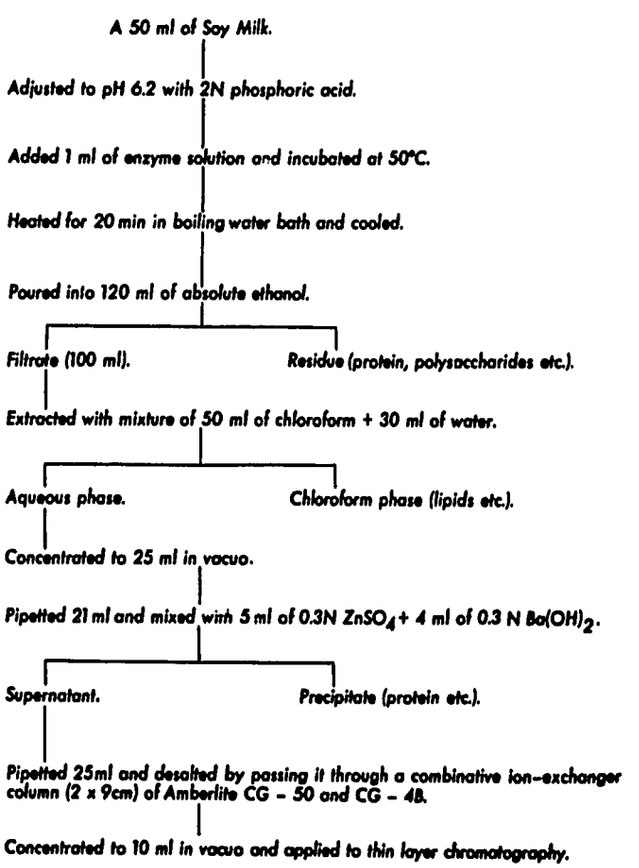

Fig. 2—Procedures for the isolation of oligosaccharides from soy milk.

preparation. Differing from the enzyme from *Aspergillus niger* (Bahl and Agrawal, 1969), the α-galactosidase in this freeze-dried preparation was stable during a storage of 2 months at room temperature. The results of enzyme assays, after each step of the purification procedures, are summarized in Table 1. The partially purified enzyme also contained β-galactosidase, α-, and β-glucosidases with specific activities (units per mg protein) of 1,800, 220,000 and 228,000, respectively. The yields of these 3 enzymes were 5.4, 7.0 and 18.2%, respectively. The enzyme preparation was completely free from protease activity.

Treatment of soy milk by enzyme

The enzymatic treatments of soy milk were performed by the following procedures: The freeze-dried soy milk powder was dissolved in distilled water containing a few drops of Antifoam A and concentration adjusted to 15% (w/v). It was then heated for 20 min in a boiling-water bath with a reflux and gentle stirring. After cooling, the pH value of the soy milk was adjusted to 6.2 from its initial 6.5 by addition of a few drops of 2 N phosphoric acid; then the final concentration of the solid material was brought to 12.0% (w/v) with distilled water.

50 ml of soy milk and 1 ml of the enzyme solution were mixed in a 100-ml Erlenmeyer flask and incubated at 50°C with a gentle reciprocal shaking for the indicated times. The enzyme reaction was stopped by placing the mixture in a boiling-water bath for 10 min. In the experiments employing pure oligosaccharides instead of soy milk as substrates, the reaction mixture was composed as follows: 10 ml of 1% (w/v) sugar solution, 9 ml of 0.04 M phosphate buffer, pH 6.2 and 1 ml of the enzyme solution.

Isolation of sugars from soy milk

50 ml of the boiled reaction mixture were poured into 120 ml of absolute ethanol and filtered. The filtrate was extracted with a tertiary solvent system according to the method of Chan and Cain (1966). The aqueous phase was concentrated in vacuo and, after removal of contaminating proteins by Somogyi's method (1945), desalted by passing it through a combination ion-exchange resin column. The neutralized eluate was concentrated and used for thin-layer chromatography. These procedures are summarized in Figure 2. In the experiments with pure oligosaccharides, the extraction procedure with the tertiary solvent system was omitted.

Thin-layer chromatography

20 μliters of the purified sugar solutions were applied to Eastman Chromagram sheets (6064, cellulose) and developed by ascending-partition chromatography using the solvent system, n-butanol-pyridine–0.1 N HCl (5:3:2 by vol) (Li et al., 1964a). When the sucrose content in soy milk was being examined, another solvent system, water-saturated n-butanol-95% ethanol-trichloroethylene (6:2:2 by vol) was employed (Chan and Cain, 1966). The latter solvent system gave a better separation of sucrose from galactose. In both cases, resolutions of sugars were enhanced by using a multiple development technique, with 4 successive developments.

To locate sugar spots on the chromatograms, 3 kinds of detectors were employed. Fructose-containing sugars were detected by dipping the chromatogram into the modified α-naphthol reagent (Albon and Gross, 1950) in which the solvent, ethanol, was replaced by acetone. Alkaline silver nitrate reagent was used to locate reducing sugars (Trevelyan et al., 1950), and diphenylamine-aniline-phosphate was applied to detect both reducing and non-reducing sugars (Buchan and Savage, 1952; Bailey and Bourne, 1960).

Estimation of oligosaccharides

The quantitative estimations of stachyose, raffinose and sucrose in soy milk were carried out by means of a so-called guide-strip technique. After the development of the sugars on the cellulose thin layer in the way described above, the chromatogram was cut into strips. The sugar spots on the guide strip were detected by the modified α-naphthol reagent. The sites of sugars on the other strips were located by reference to the guide strip. The squares containing sugars were cut out and eluted by 5 ml of distilled water in a test tube at 70°C for 2 hr with occasional shakings. After removal of cellulose powder by centrifugation, the sugar in the supernatant was estimated according to the phenol-sulfuric acid method (Dubois et al., 1956). The concentration of the sugar was calculated from standard graphs. Among standard sugar references, stachyose tetrahydrate was provided by Mr. A. Yasuda, Kikkoman Shoyu Co., Ltd., Noda, Chiba, Japan, and manninotriose by Dr. C. Y. Lee of this station.

RESULTS

Properties of α-galactosidase

Since α-galactosidase can play a most important part in the hydrolysis of soy milk oligosaccharides, some properties of the α-galactosidase in the partially purified enzyme preparation were investigated. The preparation exhibited its optimum pH between 5.0 and 5.5, but it was almost inactive below pH 2.0 and above pH 8.0 (Fig. 3). The enzyme seemed to be stable from pH 4.0–8.0; however, it

Table 2–Effect of inhibitors, metal ions, sugars and organic acids on α-galactosidase activity.

Inhibitors	Final concentration (M)	Relative activity (%)
None	–	100
KCN	1×10^{-3}	100
EDTA	1×10^{-3}	100
o-Phenanthroline	1×10^{-3}	100
8-Hydroxyquinoline	1×10^{-3}	100
Sodium thioglycolate	1×10^{-3}	100
Mercaptoethanol	1×10^{-3}	100
Cysteine	1×10^{-3}	100
p-Chloromercuribenzoate	2×10^{-5}	3
Sodium sulfite	1×10^{-3}	100
Iodine	5×10^{-4}	0
N-Bromosuccinimide	1×10^{-3}	0
Sodium arsenate	1×10^{-3}	100
Sodium fluoride	1×10^{-3}	100
Sodium pyrophosphate	4×10^{-2}	5
Metal ions		
KCl	1×10^{-3}	100
$MgCl_2$	1×10^{-3}	100
$CaCl_2$	1×10^{-3}	100
$BaCl_2$	1×10^{-3}	100
$SrCl_2$	1×10^{-3}	100
$ZnCl_2$	1×10^{-3}	35
$MnCl_2$	1×10^{-3}	91
$FeCl_3$	1×10^{-3}	49
$NiCl_2$	1×10^{-3}	100
$CoCl_2$	1×10^{-3}	100
$CuCl_2$	1×10^{-3}	3
$CdCl_2$	1×10^{-3}	91
$AgNO_3$	1×10^{-3}	0
$HgCl_2$	1×10^{-3}	0
$Pb(CH_3COO)_2$	1×10^{-3}	42
Sugars and organic acids		
D-Glucose	1×10^{-2}	85
D-Galactose	1×10^{-2}	97
D-Fructose	1×10^{-2}	100
Sucrose	1×10^{-2}	100
Sodium citrate	1×10^{-2}	62
Sodium maleate	1×10^{-2}	62
Sodium oxalate	1×10^{-2}	100
Sodium ascorbate	1×10^{-2}	100

was sensitive under more acidic conditions. During 18 hr of storage in 0.1 M glycine buffer, pH 2.5, at 30°C, about 95% of the activity disappeared.

As shown in Figure 4, the optimum temperature for the hydrolysis of melibiose was observed near 55°C. However, the enzyme itself was unstable at high temperatures. For instance, 45% of the enzyme activity was lost after the treatment at 60°C for 30 min in 0.1 M acetate buffer, pH 4.5, and it was completely inactivated by maintaining it at 70°C under the same conditions.

The effects of inhibitors, metal ions, sugars and organic acids on the reaction of the enzyme were examined using p-nitrophenyl-α-D-galactopyranoside as a substrate (Table 2). p-Chloromercuribenzoate, at a final concentration of 2×10^{-5} M caused 97% of inhibition. Therefore, the enzyme seemed to be a SH-enzyme similar in this respect to the enzymes from other origins (Hogness and Battley, 1957; Li et al., 1963; Pridham and Walter, 1964; Suzuki et al., 1966). But, from this point of view, it appeared to be somewhat different from the α-galactosidase of a higher fungus, *Calvatia cyathiformis* which is not inhibited by sulfhydryl blocking agents (Li and Shetlar, 1964b). The enzyme was also completely inhibited by the presence of 5×10^{-4} M iodine, 1×10^{-3} M N-bromosuccinimide or 4×10^{-2} M sodium pyrophosphate in the reaction mixture. Among metal ions, 1×10^{-3} M $AgNO_3$, $HgCl_2$ or $CuCl_2$ caused almost complete inhibition. Glucose at a concentration of 1×10^{-2} M caused 15% inhibition to the enzyme activity whereas galactose, at the same concentration, caused only slight inhibition. Certain organic salts such as sodium citrate or sodium maleate also caused considerable enzyme inhibition.

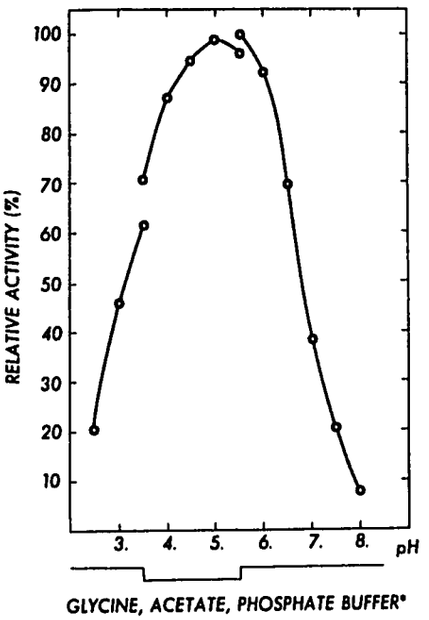

Fig. 3—Effect of pH on α-galactosidase activity. Ionic strength of all buffers in the reaction mixture is 0.1.

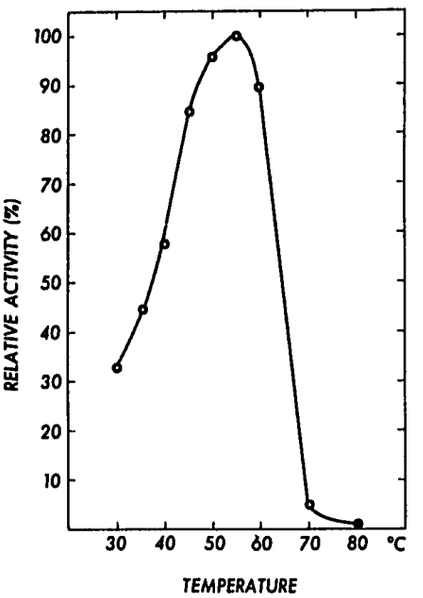

Fig. 4—Effect of temperature on α-galactosidase activity.

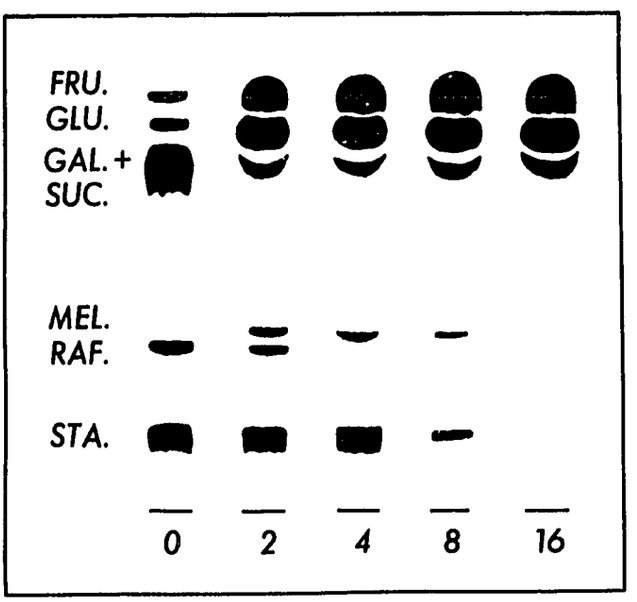

Fig. 5—Effect of the concentration of enzyme on the decomposition of oligosaccharides in soy milk. Numerals indicate the α-galactosidase activity × 10^{-3} per gram of solid material in soy milk.

Abbreviations: FRU. = fructose, GLU. = glucose, GAL. = galactose, SUC. = Sucrose, MEL. = melibiose, RAF. = raffinose, STA. = stachyose.

Detector: diphenylamine-aniline-phosphate reagent.

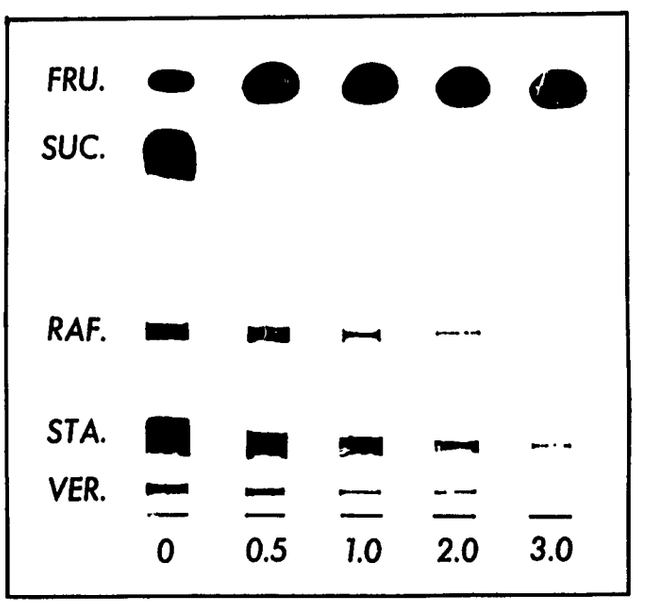

Fig. 6—Effect of incubation period of enzyme reaction on ketoses in soy milk. Numerals indicate the incubation period (hr). Abbreviations are the same as in Figure 5, except VER. = verbascose.

Detector: improved α-naphthol reagent.

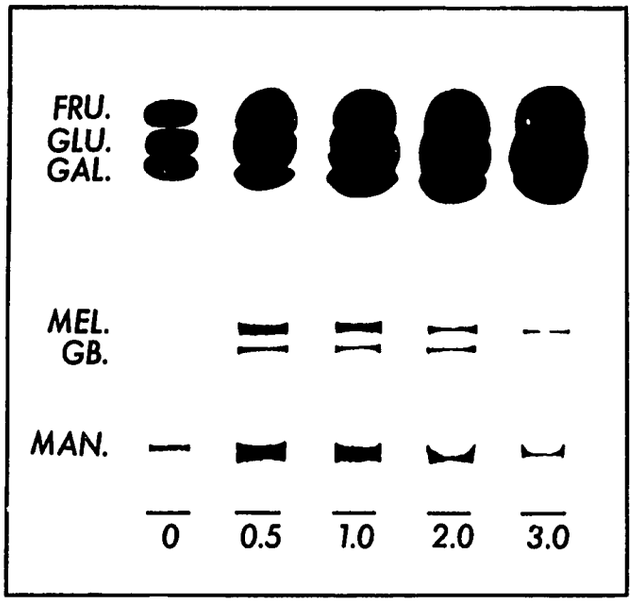

Fig. 7–Effect of incubation period of enzyme reaction on reducing sugars in soy milk. Numerals indicate the incubation period (hr). Abbreviations are the same as in Figure 5, except: GB. = galactobiose and MAN. = manninotriose.

Detector: alkaline silver nitrate reagent.

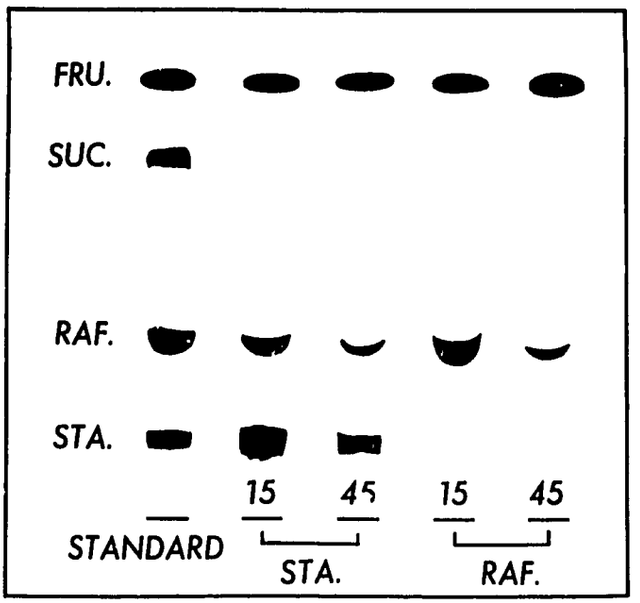

Fig. 8–Ketose-containing products of the enzymatic hydrolysis of pure stachyose and raffinose. Numerals indicate the incubation period (min). Abbreviations are the same as in Figure 5.

Detector: improved α-naphthol reagent.

Enzyme treatment was carried out as described in the text.

Lineweaver-Burk (1934) plots of the enzyme activity against the concentration of melibiose indicated a value of Km = 3.11×10^{-3} M.

The molecular weight of the α-galactosidase was determined roughly by means of a gel-filtration technique (Andrews, 1962; 1964) on a Sepharose 6B column (1.5 by 78 cm) equilibrated with a 0.05 M Tris-HCl buffer, pH 7.4, containing 0.1 M KCl. Bovine tyroglobulin, horse apo-ferritin, human γ-globulin (Fr. II), bovine serum albumin (Fr.V), egg albumen (5× cryst.) and beef chymotrypsinogen A were employed for the establishment of a standard curve for globular proteins. The elution site of the α-galactosidase was detected by its activity on melibiose. It was observed between γ-globulin and apo-ferritin; consequently, the molecular weight of the α-galactosidase was assumed to be around 290,000.

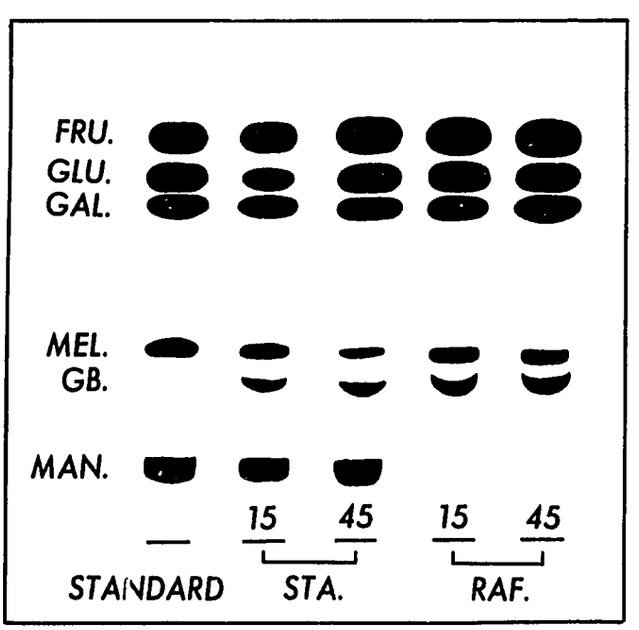

Fig. 9–Reducing sugar products of the enzymatic hydrolysis of pure stachyose and raffinose. Numerals indicate the incubation period (min). Abbreviations are the same as in Figures 5 and 7.

Detector: alkaline silver nitrate reagent.

Enzymatic hydrolysis of oligosaccharides in soy milk

Kawamura (1954) determined the contents of oligosaccharides in soybeans by means of paper chromatography and reported the following composition: sucrose 3.7%, raffinose 1.0%, stachyose 3.2% and total 7.9%. In the present experiment, as shown in Figure 5 and 6, all of these oligosaccharides could be recognized on the thin-layer chromatograms, and indicated the following contents on the dry basis of soy milk sample: sucrose 4.6%, raffinose 0.8% and stachyose 4.0% in terms of anhydrous material. Kawamura and Tada (1967) succeeded in detecting only a small amount of verbascose from Harosoy variety by means of column chromatography, but reported that pentasaccharides were not recognized on paper chromatograms. In our work, using soy milk prepared from the same variety, a sugar spot, supposedly verbascose, could be detected by the improved α-naphthol reagent on a cellulose thin-layer chromatogram (Fig. 6).

Kawamura and Tada also reported that

Harosoy variety contained free arabinose as a constitutive monosaccharide, but in our investigation no arabinose, which gives a greenish color with the diphenylamine-aniline-phosphate reagent, could be detected. However, a small amount of free fructose was observed as a constitutive monosaccharide of our soy milk sample.

As shown in Figure 5, treating the soy milk with 16×10^{-3} units of the α-galactos se preparation per gram of the solid material completely decomposed all of these oligosaccharides to their constitutive monosaccharides after 3 hr of incubation.

Figures 6 and 7 show the time course of the hydrolysis of these oligosaccharides with the same activity of the enzyme preparation. In particular, during the earlier stage of the incubation, sucrose was decomposed very rapidly by the action of invertase, and parts of both raffinose and stachyose appeared to be hydrolyzed by the same enzyme to melibiose and manninotriose, respectively.

These facts were also demonstrated in experiments using pure substrates, authentic raffinose and stachyose, as shown in Figures 8 and 9. Manninotriose, raffinose and melibiose were seen on chromatograms to be intermediates in the hydrolysis of stachyose to monosaccharides, whereas sucrose did not accumulate in detectable quantities. With regard to other possible intermediates of hydrolysis, some slight indication of the presence of α-1,6-galactobiose was present on these chromatograms.

DISCUSSION

RECENTLY, an industrial application of α-galactosidase for the improvement of crystalline sucrose recovery from beet molasses has been reported by Suzuki et al. (1963; 1964; 1969). Their research is apparently the first and the only example concerned with an application of α-galactosidase for a food manufacture. For their purpose, it was necessary to avoid contamination with invertase in the enzyme preparation. However, in removing the oligosaccharides from soy milk, the presence of invertase in the enzyme preparation can be rather useful because, on the assumption of no serious problem of accelerated browning reaction, it can promote the hydrolysis of the oligosaccharides and increase the organoleptic sweetness of the soy milk.

On the other hand, contamination with protease activity causes many undesirable effects on soy milk such as coagulation, precipitation of protein, formation of bitterness and so on; consequently, the enzyme preparation for the present purpose must be strictly free from protease activity. Fortunately, the α-galactosidase from *A. saitoi* has an especially high molecular weight. Therefore, the separation of it from protease(s) can be easily attained by means of a simple molecular sieving method. This indicates that the enzyme can be obtained very economically as a by-product of purified protease production. In addition, the particular α-galactosidase from *A. saitoi* has a few advantages. For instance, since the optimum pH for enzyme activity is in the natural pH range of soy milk, there is almost no need to change the original pH value of soy milk. Furthermore, the enzyme itself is very stable in the same pH range, and it is not necessary to take any special care in its storage. Also, differing from some of the α-galactosidases previously reported (Li and Shetlar, 1964b; Suzuki et al., 1966), the activity of the enzyme from *A. saitoi* is relatively insensitive to the presence of an end product, α-D-galactose, in the reaction mixture. The enzyme has an undesirable feature in that it is inhibited by some organic acids present in soybeans in considerable amounts (Moriguchi et al., 1961).

Separate from the results described above, there is a fundamental question; namely, whether stachyose or raffinose is actually the main cause of flatulence of soy milk (Hellendoorn, 1969). Future physiological experiments with the oligosaccharide-free soy milk will be useful in testing this hypothesis.

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