RAFFINOSE IN THE SUGARBEET (Beta vulgaris): I. BIOSYNTHESIS AND DEGRADATION IN THE ROOT; II. HYDROLYSIS IN MOLASSES WITH SWEET ALMOND EMULSIN

by

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ABSTRACT

Raffinose in the Sugarbeet (Beta vulgaris): I. Biosynthesis and Degradation in the Root; II. Hydrolysis in Molasses with Sweet Almond Emulsin

by

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Utah State University, 1970

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Department: Interdepartmental Curriculum in Food Science and Technology

PART I

The precursors for the biosynthesis of raffinose in sugarbeets were studied.

An enzyme preparation was obtained from the sugarbeet (Beta vulgaris) root. Incubation of this enzyme with \( \alpha\)-galactose-1-phosphate, Uridine triphosphate (UTP), Adenosine triphosphate (ATP), Magnesium chloride (MgCl\(_2\)), and L-cysteine at pH 5, 10\(^\circ\)C for 6 hours formed a compound which was identified as raffinose by a hydrolysis method and thin-layer chromatography. The same result was obtained when Uridinediphosphate (UDP)-galactose was incubated with sucrose, ATP, MgCl\(_2\), and L-cysteine in the presence of the beet enzyme preparation. These reactions suggested that the sugarbeet contained an enzyme system capable of transferring a galactose unit from \( \alpha\)-galactose-1-phosphate or UDP-galactose to sucrose, forming raffinose,
The galactosylation of sucrose via UDP-galactose was further confirmed using sucrose-UL-C\(^{14}\) as one of the substrates. Radioactive raffinose was identified on a radio-autograph.

The involvement of UDP-galactose in the raffinose synthesis is believed to be of more significance in vivo. A preliminary study indicated there are at least five main categories of nucleotide compounds in beet root tissues. It is suggested that a sugar nucleotide pool is present in the sugarbeet for trans-glycosylation among various sugars. A reaction similar to the above using UDP-glucose-UL-C\(^{14}\) and Nicotinamide adenine dinucleotide instead of UDP-galactose resulted in the formation of labeled raffinose. This suggested that an UDP-glucose-4-epimerase activity in beet tissues may be responsible for the conversion of UDP-glucose to UDP-galactose for the raffinose synthesis.

The possible participation of galactinol in the raffinose synthesis in sugarbeets was investigated. An experiment using galactinol, sucrose-UL-C\(^{14}\) and ATP as reactants with the sugarbeet enzyme did not give positive results.

The presence of \(\alpha\)-galactosidase activity in the beet enzyme preparation was noted. Incubation of the enzyme at 37\(^{\circ}\)C with raffinose and stachyose respectively liberated galactose as a common product. It is suggested that \(\alpha\)-galactosidase is the enzyme responsible for the breakdown of raffinose in the sugarbeet. The turnover of galactose liberated from raffinose by this hydrolytic enzyme is discussed.

The separation of \(\alpha\)-galactosidase from raffinose synthetase was attempted. Some difficulties involved are discussed.
A study of the effects of pH and temperature on the enzyme activities showed that pH 5 and low temperatures (0-15°C) are favorable for the raffinose synthesis, while high temperatures (above 15°C) favored α-galactosidase activity. Raffinose was formed when UDP-galactose and sucrose were incubated with the enzyme preparation at 0°C for 24 hours. This would account for the accumulation of raffinose during cold storage since at low temperatures α-galactosidase activity is retarded while raffinose synthetase seems to be unaffected.

PART II

Incubation of 30 milligrams commercial sweet almond emulsin (800 units/mg) with 2.5 Bx molasses which contained 1.5 mg raffinose in 3 milliliters 0.1 M acetate buffer at pH 6, 35°C for one day resulted in a complete hydrolysis of raffinose in the digest. No sucrose inversion occurred under the above conditions.
PART I

BIOSYNTHESIS AND DEGRADATION IN THE ROOT
INTRODUCTION

Raffinose is a trisaccharide composed of one molecule each of D-galactose, D-glucose, and D-fructose. It has a formula of $C_{18}H_{32}O_{16}$, and in its commercial form, has 5 molecules of water attached in crystallization. It is strongly dextra-rotatory ($[\alpha]_D^{20} = +105.2$, C4 in water).

The exact structure of raffinose: $\alpha$-d-gal$_p$-(1→6)$\alpha$-D-glu$_p$-(1→2)$\alpha$-D-fru$_f$ follows from the fact that it is completely hydrolyzed by strong acid to give galactose, glucose, and fructose. When cleaved by mild acids or invertase, D-fructose and melibiose are produced (Scheibler and Mittlemeier, 1889). The action of $\alpha$-galacto-pyranosidase results in D-galactose and sucrose (Neuberg, 1907).

Next to sucrose, raffinose is the most abundant oligosaccharide in the plant world. It occurs in high concentrations in dormant leguminous seeds and some other plant storage organs. The sugarbeet root (Beta vulgaris) contains, besides 16% sucrose, approximately 0.5% raffinose on a fresh weight basis which varies with varieties, growing areas and weather conditions and most strikingly, increases during the post harvest storage at low temperatures (Brown, 1952; Finkner et al., 1959).

Like sucrose, the raffinose in beet juices is precipitated by lime in the saccharate process and is one of the many undesirable melassigenic substances, commonly called impurities, found in beet syrup. It is undesirable because of the following reasons:
1. Raffinose has high optical activity which complicates sucrose accounting and control procedures when polarization methods are used. This is particularly true with the Steffen process where the raffinose reaches a rather high concentration due to water evaporation and sucrose crystallizations. It is not easily separated from sucrose in aqueous solution.

2. Raffinose in sufficient amounts can influence the shape of the sucrose crystal, causing it to become uneven and needle-like rather than the regular grain desired.

3. The rate of sucrose crystallization is markedly depressed as raffinose concentrations in the low raw massecuit reach 1% or more on sucrose. This is often the case at the Steffen factory, where molasses with a relatively low sucrose but rather high raffinose content from the Straight house factory is used as raw material. As a result, it is necessary to operate a Steffen factory on a continuous "molasses discard" to keep the raffinose content in the syrup within reasonable limits. This causes a slow down and economic loss to beet sugar manufacturers.

From the above discussion, it is apparent that the raffinose in sugar-beets is a problem to beet sugar processors. However, there has been only a meager amount of knowledge available in this field since Zitkowski (1911) first recognized the troublesome characteristics of raffinose and published methods of isolating it from beet sugar products. Brown (1952) and Finkner et al. (1959) both investigated the build-up of raffinose in sugarbeet roots during the cold post-harvest storage. But, the mechanism by which raffinose
accumulates in sugarbeets has not been elucidated. Therefore, there is a need to study the biosynthesis of raffinose in sugarbeets in order to provide the knowledge for controlling the raffinose level in beet juices from an industrial point of view.

This thesis presents the information demonstrating the precursors for the biosynthesis of raffinose using an enzyme preparation from the sugarbeet root. The significance of these in vitro synthetic reactions is discussed in terms of the components and metabolic patterns which have been known in the sugarbeet.

Investigations are also made on some factors which may affect the enzyme activity in question and thus the raffinose content in the sugarbeet.
REVIEW OF LITERATURE

History

Raffinose: \(\alpha\)-D-galactopyranosyl-(1\(\rightarrow\)6)-\(\alpha\)-D-glucopyranosyl-(1\(\rightarrow\)2)-\(\beta\)-D-fructofuranoside was first crystallized from Eucalyptus manna by Johnston in 1843. Later on, Loiseau (1876) isolated raffinose from beet sugar molasses and gave a correct molecular formula. But the structure of raffinose was not known until 1927 when Haworth established it by a methylation method.

Definition

Raffinose belongs to the family of raffinose oligosaccharides which are related to raffinose by virtue of having one or more \(\alpha\)-D-galactopyranosyl groups in their structures (see Table 1).

Table 1. The most common members of the raffinose family of oligosaccharides

<table>
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<th>Oligosaccharide</th>
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<tr>
<td>Melibiose</td>
<td>(\alpha)-D-gal-(1(\rightarrow)6)-(\alpha)-D-glu</td>
</tr>
<tr>
<td>Planteose</td>
<td>(\alpha)-D-glu-(1(\rightarrow)2)-(\beta)-D-fru-(6(\rightarrow)1)-(\alpha)-D-gal</td>
</tr>
<tr>
<td>Raffinose</td>
<td>(\alpha)-D-gal-(1(\rightarrow)6)-(\alpha)-D-glu-(1(\rightarrow)2)-(\beta)-D-fru</td>
</tr>
<tr>
<td>Stachyose</td>
<td>(\alpha)-D-gal-(1(\rightarrow)6)-raffinose</td>
</tr>
<tr>
<td>Verbascose</td>
<td>(\alpha)-D-gal-(1(\rightarrow)6)-stachyose</td>
</tr>
<tr>
<td>Ajugose</td>
<td>(\alpha)-D-gal-(1(\rightarrow)6)-verbascose</td>
</tr>
</tbody>
</table>
Distribution in the plant kingdom

Raffinose is the next most widespread oligosaccharide after sucrose (French, 1954). It is primarily known to occur in Taxaceae, Graminae, Myrtaceae, Chemopodiceae, Malvaceae, Tiliaceae, and Leguminae families. The seeds of food legumes usually contain raffinose in amounts equal to or greater than the amounts of sucrose. Cotton seeds and soybeans are fairly rich in raffinose and stachyose respectively (Harding, 1923; Tanret, 1913). Generally, more than one of the raffinose family oligosaccharides occur together with sucrose in the same plant. In plantago, for instance, sucrose and planteose are found in the seeds, and sucrose, raffinose and stachyose occur in the roots (Wattiez and Hans, 1943). Furthermore, these oligosaccharides may be associated with related polysaccharides such as galactomannans (characteristic of legume seeds) and sucrogalactans (characteristic of the Labiatae and allied families) (Wild and French, 1952).

Physiological functions of raffinose

Up to the present time, the physiological functions of raffinose oligosaccharides have not been completely elucidated. However, from the standpoint of plant biochemistry, these saccharides, which occur primarily in storage organs like seeds, roots and underground stems, are probably reserve carbohydrates. For example, in rice seeds, Danjo (1965) found raffinose disappeared within two days after germination. In another investigation, Rudolf and Flora (1967) reported the raffinose concentration in the embryos of Aseculus kippocartanum declined during germination. However,
recent data demonstrate the occurrence of raffinose oligosaccharides in leaves (Jeremias, 1964) where they accumulate during the winter, sometimes in the same concentration as sucrose. Under these special conditions they may serve more importantly as osmotically active substances which increase frost resistance rather than as storage materials (Kandler, 1967).

The raffinose and its accumulation in sugarbeets

The sugarbeet root contains approximately 0.05% raffinose when it is harvested. Brown and Wood (1952) demonstrated that varieties differed in their raffinose content when grown under the same conditions and concluded that these differences were due to the genotype of the varieties. They also reported that the raffinose content of the same variety could vary from location to location. For example, the sugarbeets grown in the Rocky mountain area usually had a higher raffinose content than those grown on the West coast.

Zitkowski as early as 1911 observed that the quantity of raffinose in beets increased when they were exposed to freezing weather and suggested the possibility of enzymatic action on other carbohydrates which would eventually form raffinose. Finkner and Bauserman (1956) noticed that the raffinose content of the same varieties had increased approximately 30% when harvested three weeks later in a date of harvest test. They also attributed this accumulation of raffinose to colder temperatures. Recently, Atterson et al. (1964) confirmed the above findings by stating the raffinose content of sugarbeets increased when they were left in the ground after freezing and also during prolonged storage.
An extensive investigation of the pattern of raffinose accumulation in beets during cold storage was made by Brown (1952) using a paper chromatography method of determining raffinose. He reported an increase of 0.4 ± 0.14% raffinose on sucrose, which was equivalent to an approximate 104% gain, in eight varieties of beets stored in a cellar at 8°C for a 4 months’ period. Finkner et al. (1959) using Brown’s method was able to study the varietal changes of raffinose content of beets occurring during storage and concluded:

1. No significant differences in raffinose content after a three week period between beets stored in a regular storage pile and those stored in the root cellar.

2. All varieties showed a significant increase in raffinose content during storage, however, they kept the same ranking, i.e., the varieties which had high and low raffinose content to start with also were the high and low varieties at the end of storage.

3. The rate of raffinose accumulation decreased as the beets remained in storage more than 6 weeks after harvest.

Based on the above investigation, it appears that several factors affect the raffinose content of beets. Among them are: 1. heredity, 2. environment during growth, 3. conditions of storage after harvest, and 4. interaction between these factors. This information has proved useful for sugarbeet breeders when they select mother beets for seed production.
The biosynthesis of raffinose

Judging from its structure (Fig. 1), raffinose can be imagined to arise by galactosylation of sucrose or fructosylation of melibiose. The former route for its biosynthesis was suggested by Neuberg (1907) based upon his studies on the degradation of raffinose. His hypothesis was supported by Blagoveschenski (1930) who claimed the synthesis of raffinose by the condensing effect of almond emulsin (α-galactosidase) on a mixture of sucrose and D-galactose. The small amount of product, which had $\alpha^7 D + 95.66$ in water, was hydrolyzed by the emulsin which also possesses a hydrolytic activity toward raffinose and so the product may have been at least partly raffinose. But the work should be repeated, using a more definitive test for raffinose.

Fig. 1. The structure of raffinose.

The biosynthesis of raffinose was first achieved by allowing α-phenyl galactoside to react with sucrose in the presence of α-galactosidase from the seeds of *Plantago psyllium* and *P. ovata* (Courtois et al., 1961). Pridham and
Walter (1964) later found an \( \alpha \)-galactosidase in dormant *Vicia faba* seeds which also possessed trans-galactosyl activity. Incubation of the enzyme preparation with melibiose and sucrose yielded raffinose and a small amount of plantose. In fact, it has been evident the action of hydrolytic enzymes on concentrated solutions of oligosaccharides is generally accompanied by some degree of trans-glycosylase activity in which a glycosyl group is transferred from a donor to an acceptor. In the above investigations, however, the yields of raffinose were low compared to the amount of galactose formed by hydrolysis of the donors. It is doubtful whether these enzymes have any synthetic function \textit{in vivo} (Bourne et al., 1965).

The best evidence for the synthesis of raffinose via galactosylation of sucrose \textit{in vivo} was presented by Rast et al. (1963) who used spruce twigs in their investigations of the distribution of \( ^{14} \)C in raffinose after photosynthesis in \( ^{14} \)CO\(_2\) and after assimilation of labeled glucose. They found that the glucose and fructose portion of raffinose showed an equal specific activity but galactose is less active when \( ^{14} \)CO\(_2\) was applied and more active when labeled glucose was fed. If raffinose had been synthesized from melibiose and fructose, which was also possible as mentioned previously, an unequal labeling of fructose and glucose would have been expected.

The possible involvement of sugar nucleotides in raffinose biosynthesis in plants was suggested by Bean and Hassid early in 1955 when the role of UDP-glucose in carbohydrate metabolism was just established (Leloir, 1951; Dutton and Storey, 1954). The authors, after finding that uridine diphosphate D-galactose
was the D-galactose donors in floridoside ($\alpha$-galactosyl-2-glycerol) formation, speculated that D-galactose nucleotides may have well been involved in raffinose synthesis by green plants. In the light of this hypothesis, Bourne et al. (1962) achieved a raffinose synthesis by allowing UTP and $\alpha$-galactose-1-phosphate to react with sucrose-UL-C$^{14}$ in the presence of an enzyme preparation from dormant *Vicia faba* seeds. They later (1965) demonstrated UTP-$\alpha$-D-galactose-1-phosphate uridyl transferase activity necessary for the formation of UDP-galactose in the same enzyme preparation. The evidence of the direct participation of UDP-galactose in the synthesis of raffinose was presented by Pridham and Hassid (1965) who incubated UDP-galactose-C$^{14}$ sucrose with an enzyme preparation also from dormant *Vicia faba* seeds. The compound thus formed was identified as raffinose. Gomyo and Nakamura (1966) supported the above in vitro reactions by incubating solutions of soybean enzymes with UDP-galactose and sucrose at pH 5, at 30° C, to give rise to raffinose.

Recently, Senser and Kandler (1967) and Kandler (1967) contended that galactinol (O-$\alpha$-D-galactopyranosyl-(1→1)-myo-inositol), a compound which was first isolated by Brown and Serro (1953) from sugarbeet syrup and later found in abundance in the photosynthetic leaves of the plants rich in galactosides (*Huddleia davidii*, *Thladiantha dubia* etc.), might be a galactosyl donor for the sugars of raffinose family. Evidence for such a transfer of the galactosyl group from galactinol in vitro has been obtained for the biosynthesis of stachyose with an enzyme from ripening seeds of the dwarf bean (*Phaseolus vulgaris*) (Tanner and Kandler, 1966). Kinetic studies later (Tanner and
Kandler, 1968) showed this enzyme: galactonol:raffinose-6-galactosyl transferase favored the synthesis of stachyose and was different from \(\alpha\)-galactosidase which remained in the enzyme preparation. However, no direct transfer of galactose from galactinol to sucrose to produce raffinose was observed.

As discussed previously, the sugarbeet contains 0.05% raffinose which increases during post-harvest cold storage, and yet the mechanism by which raffinose accumulates in beets has not been elucidated. As early as 1911, Zitkowski quoted professor Herzfeld in 1889 as saying that the raffinose was formed in larger quantities when beets which had been exposed to freezing weather took a new growth, in which case raffinose was probably formed from dissolved pectic substances. In the same paper, Zitkowski suggested the possibility of enzymatic action on other carbohydrates, which would eventually form raffinose, and one of these conditions was undoubtedly the action of frost. Later on, Beloal and Lemoyne (1949) thought the high content of raffinose in sugarbeets after frost may have been due to the combination of sucrose with galactose set free from galactan. But the actual galactose donors for the raffinose remained to be demonstrated.
MATERIALS AND METHODS

Materials

Sugarbeet roots of the 1101 Logan Experimental variety were harvested at the Utah State University Experimental Farm, cleaned and stored in a refrigerator at 4°C, 98% relative humidity for experimental use. The microbial growth of beets during the storage period was inhibited by ultraviolet light.

Chemicals

All the chemicals used were of C. P. or equivalent grade.

Adenosine triphosphate (ATP), uridine triphosphate (UTP), α-galactose-1-phosphate (gal-1-p), uridyl diphosphate galactose (UDP-gal) and L-cysteine were obtained from Sigma Chemical Company, St. Louis, Missouri.

Sucrose-C\(^{14}\) (13.5 μc per μ mole, uniformly labeled) was obtained from New England Nuclear Corporation, Boston, Massachusetts.

Silica Gel G with gypsum binder for thin-layer chromatography was purchased from Warner-Chilcott Laboratories Instruments Division, Richmond, California.

Phenol reagent (2N) for the protein determination, D-fructose and ammonium sulfate were obtained from Fisher Scientific Company, Fair Lawn, New Jersey.

p-anisidine·HCl was purchased from Eastman Kodak Company, Rochester, New York.
Raffinose was obtained from Mann Research Laboratory, New York, New York.

L-ascorbic acid, n-butanol, hydrochloric acid, glacial acetic acid, EDTA, sodium acetate, magnesium chloride and mercuric oxide were products of J. T. Baker Chemical Company, Phillipsburg, New Jersey.

Galactinol (myo-inositol-\(\alpha\)-D-galactopyranoside) dihydrate, "A" grade was product of Calbiochem, Los Angeles, California.

Protein determination

The method of Lowry et al. (1951) was used.

Enzyme preparation

The procedure was carried out in a cold chamber (4-10\(^\circ\)C). Roots were washed with water, peeled and cut into small segments. One hundred grams of the tissue were homogenized in a Waring blender with 125 ml cold 3\% sodium acetate solution containing 5 \times 10^{-5} M EDTA and 500 mg L-ascorbic acid which was used as an antioxidant to retard the darkening of the juice. After 2 minutes of homogenation the liquid portion was rapidly filtered through cheese cloth and then filtered through glass wool to yield ca. 150 ml of crude extract, which was immediately centrifuged at 20,000 x G for 20 minutes. The precipitate was discarded and the supernatant was saturated with powdered \((\text{NH}_4)_2\text{SO}_4\). The solution was allowed to stand in a refrigerator for 1 hr., and then centrifuged again at 20,000 x G for 20 minutes. The supernatant was discarded and the precipitate was taken up in a minimum
amount of 0.05 M acetate buffer (pH 5) and dialyzed against the same buffer at 5°C for 24 hrs. Approximately 20 ml of enzyme solution (containing ca. 30 mg protein), free from sugars and nucleotides, was obtained. The extract was used within 24 hrs, since the activity diminished on storage at 5°C. Freezing caused coagulation of the enzyme and complete loss of the raffinose synthesizing activity.

**Synthesis of raffinose**

The enzymes (15 mg protein in 0.5 ml of 0.05 M acetate buffer, pH 5) were incubated with ATP (5 μ moles), UTP (3 μ moles), α-D-galactose-1-phosphate (5 μ moles), sucrose 10 μ moles), MgCl₂ (10 μ moles) and L-cysteine (3 μ moles) at 0°C, 10°C and 37°C, respectively.

Identical reaction mixtures except (a) with boiled enzymes, (b) with UDP-gal (5 μ moles) instead of UTP and α-D-gal-1-phosphate and (c) with U-C¹⁴-sucrose (10 μc, 10 μ moles) instead of regular sucrose were also run.

The reactions were terminated by boiling the reaction mixtures for 30 seconds after a set period of time (e.g., 0 hr., 1 hr., 3 hrs., 6 hrs., 12 hrs., etc.). The precipitate was removed by centrifugation.

**Thin-layer chromatography**

Twelve grams of silica gel G were mixed with 27.6 ml 0.02 M pH 8.0 borate buffer by stirring for ca. 60 seconds. The slurry was coated on 2 glass plates (20 cm x 20 cm) using an applicator. The plates were kept at room temperature until they set and then dried for 2 hrs. at 110°C. They were
stored in a desiccator at room temperature before use.

A micropipet was used to spot the samples, and a warm stream of air from a hair dryer was used to dry the spots. The application was carried out by spotting about $10\lambda$ ($10^{-6}$) each time, drying and repeating this procedure until the desired volume (usually $30\lambda$) was put on the plate. The origin was 2 cm above the bottom edge of the plate. The thin layer was broken vertically 2 cm from each side to eliminate edge effects.

The plate was allowed to develop by ascending chromatography to a height of 18 cm in closed glass tanks containing 1-butanol-acetic acid-water (5:4:1) as solvent system. The average development time at $20^\circ$C was 3 hrs. The plate was dried in a hood followed by in oven at $70^\circ$C for 15 minutes and cooled to room temperature. It was returned to the solvent tank for a second run, and then, after being dried as before, sprayed with p-anisidine phosphate reagent (1% p-anisidine HCl in 100 ml EtOH + 10 ml orthophosphoric acid).

The sprayed plate was heated for 15 minutes at $110^\circ$C. The sugars appeared as brown spots on a light yellow background. The colors were only stable in the dark at room temperatures.

**Separation of carbohydrates**

The clear reaction mixtures containing sugars were spotted on a thin layer plate. After two successive developments, sugars were located by spraying the plate with p-anisidine phosphate reagent or by placing the chromatogram in contact with Kodak No-Screen X-ray film for 3-7 days to obtain radioautograms when sucrose-UL-C$^{14}$ was used as a substrate.
Identification of raffinose (R)

1. The reaction mixture was applied as a line on a thin layer plate, which was developed and dried according to the method described. The band containing compound R on the unsprayed plate was located by referring the R of the spot of standard raffinose on an index plate which was developed under the same conditions as the above but sprayed with p-anisidine reagent. The silica gel of the band corresponding to the position of standard raffinose was scraped off the glass plate, collected with suction and placed in a test tube. The sugar adhering on the silica gel particles was extracted with 80% ethanol three times. The extraction was evaporated to dryness, subjected to hydrolysis with 2N HCl (0.1 ml) at 100°C for 3 hrs., neutralized with 2N NaOH and the sugars identified by thin layer chromatography.

2. Authentic raffinose was added to either sample solutions or extractions obtained as (1) followed by co-chromatography on thin layer plates.

Extraction of nucleotides from sugarbeet roots

The procedure for the isolation of the sugarbeet root nucleotides was a modification of the method of Ginsburg et al. (1956) used for the isolation of nucleotides from Mung bean seedlings.

Ten kgs of sugarbeet roots were homogenized in 10 liters of 95% ethanol by means of Waring blendors. The suspension was heated to boiling, allowed to stand overnight at room temperature, and then centrifuged. The supernatant liquid was squeezed through cheese cloth, acidified with 5 N nitric
acid to Ph 2.5, and 240 ml mercuric acetate solution\(^1\) was added (Caputto et al., 1950). After cooling to \(0^\circ C\), the resulting precipitate was centrifuged. The precipitate was suspended in 1 liter of water, and a few drops of octyl alcohol were added, after which hydrogen sulfide was bubbled through the suspension for 4 hours with vigorous stirring. The solution was centrifuged and the supernatant liquid was aerated for 1 hr. and neutralized to pH 7 with concentrated ammonium hydroxide. The entire operation was carried out at \(0^\circ C\).

**Anion ion exchange chromatography**

The neutralized solution was passed at room temperature through a column of Dowex 1 resin in the chloride form (2% cross-linked, 50 to 100 mesh) measuring 4.5 cm in diameter and 35 cm in height. The column was washed with distilled water until the optical density of the eluate at 260 \(\text{m} \mu\) fell below 0.1. The column was then eluted with 0.01 N hydrochloric acid containing increasing concentrations of sodium chloride. The elutiation rate was approximately 10 ml per minute, and the eluate was collected in 500 ml fractions by means of an automatic fraction collector (Ginsburg, 1956). Fig. 2 shows the set up of such a collector. A total volume of 40 liters was collected using this apparatus. Elution of the nucleotides was followed by changes in optical density of the eluate at 260 \(\text{m} \mu\).

\(^1\)The mercuric acetate solution was prepared by mixing 13.5 gm of yellow mercuric oxide, 9.2 ml of glacial acetic acid, and water to make 100 ml.
Fig. 2. Large scale automatic fraction collector for the ion exchange chromatography of sugar nucleotides.
RESULTS AND DISCUSSION

Raffinose is a constituent of saccharides in sugar beets, where it accumulates during post-harvest cold storage (Zitkowski, 1911; Finkner and Bauserman, 1956). In order to investigate the pathway by which raffinose is synthesized in sugar beets, an enzyme extract was prepared from the stored root portion of the plant according to the method described previously. Incubation of the enzyme preparation with \( \alpha \)-D-galactose-1-phosphate (5 \( \mu \) moles), sucrose (10 \( \mu \) moles), UTP (3 \( \mu \) moles), ATP (5 \( \mu \) moles), MgCl\(_2\) (10 \( \mu \) moles) and L-cysteine (3 \( \mu \) moles) at pH 5, 10\( ^\circ \)C for 6 hrs. produced compound R\(_1\) with the mobility on a thin-layer chromatogram identical to that of authentic raffinose. The control using a boiled enzyme gave no corresponding reaction product. Fig. 3 shows the locations of various sugars resulting from the incubation on a thin-layer chromatogram, \( C_1 \) and \( S_1 \) referring to the control and the reaction mixture respectively. For confirmation of the compound R\(_1\), authentic raffinose was added into the reaction mixture and then subjected to thin-layer chromatography. A coincidence of compound R\(_1\) and authentic raffinose on the chromatogram was observed (see Fig. 4). Compound R\(_1\) extracted with 80% ethanol from an unsprayed thin layer plate was hydrolyzed with 2 N HCl at 100\( ^\circ \)C for 3 hrs. This hydrolysis produced melibiose and fructose from raffinose which were identified by thin-layer chromatography. The results thus obtained showed that compound R\(_1\) was raffinose, and also indicated that galactose-1-phosphate...
Fig. 3. Thin-layer chromatogram of products formed in raffinose synthesizing systems.

C₁: Control of S₁; S₁: Reaction mixture of the incubation of α-galactose-1-phosphate, sucrose, UTP and ATP with the sugar beet enzyme preparation at 10°C, pH 5 for 6 hours.

C₂: Control of S₂; S₂: same as S₁ except using UDP-galactose instead of α-galactose-1-phosphate and UTP as a substrate.

R: position of compounds R₁ found in S₁ and R₂ found in S₂.

Standards:
A: raffinose; B: sucrose; C: fructose; D: galactose; E: glucose.

The thin-layer chromatogram was stained with p-anisidine phosphate reagent and heated at 110°C for 15 minutes.
Fig. 4. Coincidence of compound $R_1$ and authentic raffinose on a thin-layer chromatogram.

$S_1$: Reaction mixture containing compound $R_1$.

$A$: Authentic raffinose.

$S_1 + A$: Reaction mixture plus authentic raffinose.

$R_1$: Synthetic raffinose.
can serve as a galactose source in the raffinose synthesis by the sugarbeet enzyme extract. An attempt to determine compound R₁ quantitatively using the galactose oxidase method (Avigad et al., 1961) was not successful because the amount of the sugar was too small.

In another experiment, UDP galactose was incubated with sucrose and ATP in the presence of the sugarbeet enzyme preparation, giving rise to compound R₂ (Fig. 3), which was further identified as raffinose by co-chromatography and acid hydrolysis methods described in the above. This reaction showed the enzyme preparation is also capable of catalyzing an enzymatic transfer of D-galactose from UDP-galactose to sucrose to form raffinose in vitro. This enzyme activity is consistent with what has been found in *Vicia faba* seeds and soybeans (Pridham and Hassid, 1965; Gomyo and Nakamura, 1966).

The involvement of UDP-galactose in the reaction is believed to be of more significance since this sugar nucleotide is undoubtedly widely spread in plant tissues (Ginsburg et al., 1956; Brown, 1962) and by analogy with sucrose synthetase action, the equilibrium constant for the reaction should favor raffinose synthesis in vivo (Cardini et al., 1955; Mendicino, 1960).

A preliminary study indicated there are at least five main categories of nucleotide compounds in beet root tissues (Fig. 5), with characteristic maximum absorption at wave length 260 μ (Fig. 6). In sugarbeet tissues there should be a nucleotide pool responsible for trans-glycosylation among various sugars according to the present concept of carbohydrate metabolism. Avigad (1966) isolated UDP-galactose from sugarbeet tissues and also found
Fig. 5. Separation of sugarbeet nucleotides by anion exchange chromatography using a Dowex 1 x 2 (Cl⁻) column, 50-100 mesh. The nucleotides were eluted with 0.01 N hydrochloric acid containing increasing concentrations of sodium chloride as indicated.
Fig. 6. Ultraviolet absorption spectra of sugarbeet nucleotides separated by anion exchange chromatography using a Dowex 1 (Cl⁻) column. Peak I, II, and III refer to the fractions of eluate collected under the same peaks shown in Fig. 5.
UDP-glucose-4-epimerase activity which catalyzes the conversion of UDP-glucose, a direct degradation product of sucrose during respiration, according to the author, into UDP-galactose. From his findings, the author suggested that the formation of sugar nucleotides from sucrose can be considered as a supplier of substrates for further biosynthetic reaction, such as the formation of complex glycosides and polysaccharides in the tissue. This hypothesis seems applicable for the UDP-galactose dependent synthesis of raffinose in sugarbeets. A raffinose synthesis reaction was carried out using UDP-glucose-UL-C$^{14}$, sucrose, NAD (3 μ moles), ATP, MgCl$_2$ and L-cysteine as reactants to incubate with the sugarbeet enzyme preparation. In the reaction mixture labeled raffinose was identified by thin-layer chromatography. The result indicated that an UDP-glucose-4-epimerase activity in sugarbeet tissues may be responsible for the conversion of UDP-glucose to UDP-galactose for the raffinose synthesis. It is possible that nucleotide diphosphates other than UDP-galactose and UDP-glucose are involved. This obviously requires further investigation.

In order to further confirm the step involving galactosylation of sucrose by UDP-galactose in raffinose formation, sucrose-UL-C$^{14}$, UDP-galactose and ATP were incubated with the enzyme preparation at pH 5, 10°C, followed by thin-layer chromatography. A radio-autograph (Fig. 7) shows the formation of a labeled compound in reaction mixtures of different incubation times with chromatographic mobility identical to that of authentic raffinose. It is noticeable the radioactivity of the labeled compound remains nearly
Fig. 7. Radio-autograph of thin-layer chromatogram of reaction mixtures after the incubation of UDP-galactose, sucrose-UL-C^{14} and ATP with the sugarbeet enzyme preparation at pH 5, 10°C for 0.5, 1, 3, 6, and 12 hrs.

R: Synthetic raffinose; Suc: Sucrose.
F: Fructose.
unchanged after 1 hr. of incubation. This suggests that the raffinose formation reaches an equilibrium state rather fast when the crude enzyme is used. Kinetics studies on the raffinose synthetase should be based on a pure enzyme preparation.

Galactinol (O-\(\alpha\)-(D-galactopyranosyl-(1→1)-myo-inositol), a compound first isolated by Brown and Serro (1953) from sugarbeet syrups was shown to be a galactose source in stachyose biosynthesis in the dwarf bean (Tanner and Kandler, 1966). The concentrations of galactinol in some varieties of sugarbeets were as high as those of raffinose at the time of harvesting (McAllister et al., 1961). The possibility of galactinol acting as a galactose source in the raffinose synthesis cannot be neglected. A preliminary experiment using galactinol, sucrose-UL-C\(^{14}\) and ATP as reactants with the sugarbeet enzyme preparation indicated the formation of a labeled compound with mobility on a radio-autograph of thin-layer chromatogram similar to that of stachyose. However, only trace amount of labeled raffinose was formed in the same reaction mixture. These results suggest that galactinol does not play an important role in the biosynthesis of raffinose in sugarbeets.

The presence of \(\alpha\)-galactosidase activity in the beet enzyme preparation was observed. Incubation of the beet enzymes at 37\(^0\)C with raffinose and stachyose respectively liberated galactose as a common hydrolytic product (Fig. 8). The galactose set free in the hydrolysate was easily distinguished from glucose and fructose on thin-layer chromatogram using borate-impregnated silica gel G plate and a mixture of 1-butanol-acetic acid-water (5:4:1) as the
Fig. 8. Thin-layer chromatogram of the hydrolytic products resulting from incubation of raffinose and stachyose respectively with the sugarbeet enzyme preparation (pH 5) at 10°C for 1 and 6 hrs.

\( R_a \) : Raffinose as substrate.

\( S_t \) : Stachyose as substrate.

Standards: A: raffinose; B: galactose; C: fructose.

developing system. In fact, $\alpha$-galactosidase activity has been widely detected in the dormant seeds of coffee, cotton, and *Vicia faba*, which contain high concentrations of raffinose and stachyose (Shiroya, 1963; Shadaksharaswamy and Ramachandra, 1968; Dey and Pridham, 1968). It has also been observed during soaking and germination $\alpha$-galactosidase activities in the above tissues increased, while raffinose and stachyose declined rapidly and finally disappeared completely in some cases. Strangely enough, no free galactose was detected throughout all stages of germination. Shiroya (1963), however, was able to find an enzyme system, probably galactose kinase, which rapidly utilized galactose in the intact cotyledons of germinated cotton seedling. He suggested that the galactose liberated from raffinose by the action of $\alpha$-galactosidase could be metabolized *in vivo* through its UDP derivative which was formed following the phosphorylation of free galactose. This speculation explains well the phenomena of sugar transformations in stored sugarbeets observed by McCready and Goodwin (1966). They reported the raffinose concentrations fell off rapidly during storage at $25^\circ\text{C}$ but no galactose or melibiose were detected in root tissues. The authors suggested that raffinose broke down under these conditions into sucrose and UDP-galactose which were further metabolized. Free galactose never accumulates in the beet tissue. Up to now it appears that the role of $\alpha$-galactosidase in plant tissues is mainly in hydrolyzing the raffinose family of compounds during growth periods or when localized raffinose concentrations reach an excess, rather than transglycosylation for the synthesis of galactosidic bonds. A decrease in the accumulation rate of raffinose in
stored beets when the storage period was prolonged (Finkner et al., 1959) should be attributed to the rising $\alpha$-galactosidase activity in those tissues and not to lack of substrates for raffinose synthesis.

The accompaniment of $\alpha$-galactosidase with the raffinose synthesizing system makes it difficult to study the biosynthesis of this oligosaccharide using a crude enzyme preparation. So far the "raffinose synthetase" has not been isolated and studied in a purified state. It is because the similar physical and chemical properties of $\alpha$-galactosidase and raffinose synthesizing enzyme make a separation very difficult. Attempts to effect a transfer of the galactosyl moiety of UDP-galactose to sucrose for the formation of raffinose with an enzyme preparation from mung bean seedlings was not successful. If raffinose was formed, the germinated seedling most probably contained $\alpha$-galactosidase which hydrolyzed this oligosaccharide (Hassid, 1969).

In this investigation, the synthesis of raffinose was only achieved by incubating the reaction mixtures at optimum conditions for the synthesizing enzyme system from sugarbeets (i.e., pH 5, temperature 0-15°C). At conditions of pH 7 and a temperature of 37°C, no amount of raffinose detectable by thin-layer chromatography was formed. An attempt to separate the raffinose synthetase from $\alpha$-galactosidase from sugarbeets by ammonium sulfate fractionation was not successful. This may have been due to the similar affinity toward water of these two enzymes in buffered aqueous solution. It was also noted that low temperatures (even as low as 0°C) favor the synthesis. Fig. 9 shows the raffinose formed when UDP-galactose and sucrose were
Fig. 9. Thin-layer chromatogram of products formed by incubating UDP-galactose and sucrose with the beet enzyme preparation at 0°C, pH 5 for 24 hrs.

C: Control  
S: Reaction mixture  
A: Authentic raffinose  
S+A: Reaction mixture + Authentic raffinose  
B: Standard sucrose  
R: Raffinose synthesized
incubated with the enzyme preparation at 0°C for 24 hrs. This would account for the accumulation of raffinose during cold storage since at low temperatures α-galactosidase activity is retarded while the raffinose synthetase seems to be unaffected.

In summary, the biosynthesis of raffinose in sugarbeets can be represented by the following scheme (Fig. 10):

Fig. 10. The proposed pathway for the synthesis of raffinose in sugarbeets.

The sugar nucleotide dependent pathway would also allow the raffinose synthesis in sugarbeets to be closely linked to sucrose metabolism.
SUMMARY AND CONCLUSION

The precursors for the biosynthesis of raffinose in sugarbeets were studied.

An enzyme preparation was obtained from the sugarbeet (Beta vulgaris) root. Incubation of this enzyme with $\alpha$-galactose-1-phosphate, UTP, sucrose, ATP, MgCl$_2$ and L-cysteine at pH 5, $10^0$C for 6 hrs. formed a compound which was identified as raffinose by a hydrolysis method and thin layer chromatography. The same result was obtained when UDP-galactose was incubated with sucrose, ATP, MgCl$_2$ and L-cysteine in the presence of the beet enzyme preparation. These reactions suggested that the sugarbeet contained an enzyme system capable of transferring a galactose unit from $\alpha$-galactose-1-phosphate or UDP-galactose to sucrose, forming raffinose. The galactosylation of sucrose via UDP-galactose was further confirmed using sucrose-UL-C$^{14}$ as one of the substrates. Radioactive raffinose was identified on a radio-autograph.

The involvement of UDP-galactose in the raffinose synthesis is believed to be of more significance in vivo. A preliminary study indicated there are at least five main categories of nucleotide compounds in beet root tissues. It is suggested that a sugar nucleotide pool is present in the sugarbeet for trans-glycosylation among various sugars. A reaction similar to the above using UDP-glucose-UL-C$^{14}$ and NAD instead of UDP-galactose resulted in the formation of labeled raffinose. This indicated that an UDP-glucose-4-epimerase activity
in beet tissues may be responsible for the conversion of UDP-glucose to UDP-galactose for the raffinose synthesis.

The possible participation of galactinol in the raffinose synthesis in sugarbeets was investigated. An experiment using galactinol, sucrose-UL-C\textsuperscript{14} and ATP as reactants with the sugarbeet enzyme did not give positive results.

The presence of $\alpha$-galactosidase activity in the beet enzyme preparation was noted. Incubation of the enzyme system at 37°C with raffinose and stachyose respectively liberated galactose as a common product. It is suggested that $\alpha$-galactosidase is the enzyme responsible for the breakdown of raffinose in the sugarbeet. The turnover of galactose liberated from raffinose by this hydrolytic enzyme is discussed.

The separation of $\alpha$-galactosidase from raffinose synthetase was attempted. Some difficulties involved are discussed.

A study of the effects of pH and temperature on the enzyme activities showed that pH 5 and low temperatures (0–15°C) are favorable for the raffinose synthesis, while high temperatures (above 15°C) favored $\alpha$-galactosidase activity. Raffinose was formed when UDP-galactose and sucrose were incubated with the enzyme preparation at 0°C for 24 hrs. This would account for the accumulation of raffinose during cold storage since at low temperatures $\alpha$-galactosidase activity is retarded while raffinose synthetase seems to be unaffected.
PART II

HYDROLYSIS IN MOLASSES WITH SWEET ALMOND EMULSIN
INTRODUCTION

In the manufacture of sucrose from sugarbeets (Beta vulgaris) only about 90% of the sucrose which the beets contain can be recovered unless special purification processes are carried out to remove the non-sucrose impurities from the crystallization mixtures. When the purity, i.e., the percentage ratio of sucrose to total solids drops to 70%, the rate of crystallization of sucrose becomes so slow that continuation of the process becomes economically unsound. Among the most frequently suggested impurities responsible for a decrease in sucrose crystallization rate are raffinose, gums such as pectins and dextrin, protein and other nitrogen compounds and inorganic salts. It has been found that the very slowly crystallizing syrups usually contain rather high concentrations of carbonate and chlorate salts and the decomposition products of glutamine (Rorabaugh and Norman, 1956). High raffinose is also associated with difficultly crystallizable syrups (Van Hook, 1946). However, the physical phenomena by which these impurities control the rate of crystallization of sucrose from syrups are not well understood.

Raffinose (\(\alpha\)-D-galactose(1→6)-\(\alpha\)-D-glucose(1→2)-\(\alpha\)-D-fructoside), a trisaccharide naturally occurring in sugarbeets, is one of the most undesirable impurities and interferes with the recovery and refining of sucrose. It has been described previously. From his work on crystallization of sucrose, Hungerford (1942) reported that raffinose changed the relative growth rate of sucrose crystal
faces, resulting in a typical distorted crystal from syrups containing more than 1% of the trisaccharide on sucrose. At $40^\circ$C, 90% purity, the time required to produce a crystal of given weight is nearly 60% longer in the presence of 1.03% than in the presence of 0.44% raffinose. The actual mechanism by which raffinose causes the slowdown of sucrose crystallization rate has not been established. The work of Mariani and Ciferri (1954) indicated that the effect of raffinose on crystallization rate was not due to an increase in the solubility of sucrose in the presence of this trisaccharide. On the contrary, their results showed a decrease in the solubility of sucrose when raffinose was present in a solution saturated with both solutes and also that sucrose was slightly less soluble under these conditions at higher temperatures. Recently, Dunning et al. (1965) recognized that added raffinose and stachyose affected a rate controlling process taking place at or on the surface of sucrose crystals probably by adsorption. They suggested that the chemical structure of impurities seemed to be a significant factor since raffinose and stachyose could form a strong fructose-fructose bond with the surface of sucrose crystals, while other saccharides (i.e., melextose) would only form weak glucose-glucose bonds with the surface. Motitsugu (1967) found a concentration of 0.6% raffinose reduced the rate of sucrose crystallization from a super-saturated sucrose solution by 55% apparently by being adsorbed on the surface of the sucrose crystals.

From the above discussion, it appears that a suitable method to reduce or eliminate the raffinose impurity in sugar syrups should increase the yield of
sucrose from beets and would thus be beneficial to beet sugar manufacturers. Raffinose, like sucrose, is precipitated by lime in the saccharate process and is mostly retained in the syrups because of continued recycling of the juices in the sugar factory. It is, therefore, impossible to separate this trisaccharide from sucrose in the factory with the processing methods used today. However, an area in the utilization of enzymes in purifying beet syrups is believed to be feasible since the enzyme specificity should provide selectivity in eliminating raffinose leaving sucrose unaffected. For example, decomposition of raffinose by $\alpha$-galactosidase of actinomyces, penicillium and streptomyces during fermentation has been reported (Suzuki et al., 1966a; Suzuki et al., 1966b; and Rounds, 1969).

In this investigation, sweet almond emulsin which contains an $\alpha$-galactosidase capable of splitting the $\alpha$-galactosyl(1$\rightarrow$6)$\alpha$-glucosidic bond to liberate galactose from raffinose leaving sucrose was initiated to study the purification of raffinose containing beet molasses. $\alpha$-galactosidases are widely found in nature. The reasons for using sweet almond emulsin are: 1. Almond emulsin is a classical source of high $\alpha$-galactosidase activity from plants (Zechmeister et al., 1938); 2. It is commercially available in large quantities in crude form. Recently, a pure sweet almond $\alpha$-galactosidase has been prepared (Malhotra and Dey, 1967a). Studies upon the specificity of this enzyme indicated it has an optimum pH at 5.5-5.7, $K_m$ $12.5 \times 10^{-3}$, $V_{max}$ 11.8 (u moles min$^{-1}$mg$^{-1}$), no feedback inhibition and works well using raffinose as substrate (Malhotra and Dey, 1967b).
In the course of studying the effect of almond emulsin in removing
the raffinose impurity, molasses obtained from two local sugarbeet factories
was incubated with the enzyme. Changes of important sugars in the digests were
examined at intervals by paper chromatography. Optimum conditions for the
reaction in terms of pH, incubation time and temperature were also elucidated.
It is hoped that the results may provide some basis for the use of an enzymatic
process to eliminate the reaaffinose in sugar syrups.
MATERIALS AND METHODS

Materials

1. Molasses for experimental uses was obtained from Amalgamated Sugar Company plant at Lewiston, Utah, and Utah-Idaho Sugar Company plant at Garland, Utah, during the campaign of December, 1965.

2. Sweet almond emulsin lyophilized, salt free, with an activity of 800 units/mg was purchased from Mann Research Laboratory, New York, New York, prepared according to the method of Hestrin et al. (1955). One unit of activity is that liberating one micromole of galactose per minute from α-phenyl galactoside at pH 5, 30°C.

Methods

Measurement of ultraviolet absorption of the enzyme. The enzyme sweet almond emulsin was dissolved in water and its optical densities were measured at different wave lengths from 240-350 μm. An absorption spectrum was obtained by plotting optical density against wave length.

Preparation of samples. Molasses obtained from the factory was diluted to 5°Bx with water for experimental use.

Determination of raffinose in molasses solution. A modified method of Jantzef and Potter's (1964) was used.
a. Raffinose standard (30 μg/μl anhydrous raffinose)

3.54 g raffinose pentahydrate was dissolved in 100 ml water. The stock and standard solutions were preserved by adding a few crystals of thymol and storing in a refrigerator.

b. Chromatographic solvent

1-Butanol-acetic acid-water (4:1:5) upper layer was used for development.

c. Coloring reagent

Two g p-anisidine was suspended in about 40 ml absolute ethanol dissolved by adding 10 ml concentrated hydrochloric acid and diluted to 100 ml with absolute ethanol.

d. Chromatographic paper

Whatman No. 1, chromatographic grade paper (18 1/2 x 22 1/2 inches).

e. Sample treatment

Five ml of 5°Bx molasses solution was transferred to a 25 ml volumetric flask with water. One ml saturated neutral lead acetate solution was added. This quantity precipitated interfering substances of most samples. The sample was mixed, diluted to volume and added one drop of amyl alcohol if foam hindered dilution. It was allowed to stand for 30 minutes then filtered with Büchner funnel.
f. Paper chromatography

Standards (20r, 40r, 60r, 80r, 100r) and sample were spotting in a volume of 10 μl on chromatographic sheets at 1.5 cm intervals. The sugars were separated by descending chromatography. After 64 hour development the sheets were air dried. Each sheet was dipped by drawing it through a trough containing p-anisidine reagent. The sheet was air dried in a hood and heated in a forced air oven for 5 minutes at 110°C. After allowing it to stand for a few minutes at room temperature, the color of the developed raffinose spots was measured with a transmission densitometer (Photovolt Corporation, New York, New York).

A standard curve was obtained by plotting reading against raffinose concentrations.

Incubation of samples with sweet almond emulsin. Raffinose, sucrose (30 mg each) and 5°Bx molasses solution (1.5 ml) were incubated with 20 mg sweet almond emulsin in 3 ml (1.5 ml in the case of molasses) of 0.2 M acetate buffer (pH 5-8) respectively. At the end, the reaction was stopped by adding one volume of ethanol and protein removed by centrifugation. Sugars in the digests were examined at intervals by paper chromatography, the results being expressed as follows: ++ end product in large quantity, + end product in moderate quantity, - not detectable. By this method, reproducible results have been obtained. Unless otherwise stated, enzymatic reactions were carried out at 35°C, aseptic conditions being maintained by use of toluene.
Paper chromatography for identifying sugars in digests.

a. Chromatographic paper

Whatman No. 1, chromatographic grade paper.

b. Developing solvent system

A. n-butanol-acetic acid-water (4:1:5) for separating raffinose sucrose and fructose.

B. Collidine saturated with water for separating galactose and glucose

c. Coloring reagent

A. 3% χ-naphthol in acetone for locating raffinose, sucrose, and fructose.

B. p-anisidine for locating galactose and glucose.

C. Anisidine phosphate for locating galactose and glucose.

Descending paper chromatography was carried out at room temperature for at least 40 hrs., followed by spraying the air dried sheet with coloring reagents and heating at 110°C for 5 minutes.
RESULTS AND DISCUSSION

Commercial sweet almond emulsin with an $\alpha$-galactosidase activity of 800 units/mg was used in the study. An ultraviolet absorption spectrum with a peak at 280 nm and a ratio of optical density 280/260 = 1.34 indicated the purity of the enzyme (Fig. 11). The raffinose concentration in $5^\circ B_X$ molasses solution was found to be 0.1%. Fig. 12 shows the standard curve for the raffinose determination using a combination of paper chromatography and densitometry (Jantzef and Potter, 1964).

In order to determine the effects of the enzyme on raffinose and sucrose during hydrolysis, some preliminary experiments were conducted. Incubation of pure raffinose (30 mg) with the enzyme in 3 ml of 0.2 M acetate buffer (pH 5) at $35^\circ$C, followed by examination of sugars in digests every 24 hours revealed that pure raffinose at a concentration of 10 mg/ml (a 1% solution) was completely hydrolyzed by almond emulsin (10 mg/ml) at the end of 1 day (24 hours). The sucrose liberated from raffinose was further hydrolyzed into fructose and glucose (Table 2). When the incubation was continued until the fourth day, sugar varieties in digests remained the same as in the first day digest. In Table 3 are the results of incubation at pH 6 under the same conditions as above except the pH. It is evident that the enzyme activity decreased as compared to that at pH 5. A complete hydrolysis of raffinose occurred at the end of a 2 day incubation (48 hours). Table 4 indicates the same trend of decreasing enzyme activity at pH 7. As a result,
Fig. 11. Ultraviolet absorption spectrum of sweet almond emulsin.
Fig. 12. Standard curve for the raffinose determination. Raffinose of different concentrations was spotted on a chromatographic paper, developed with n-BuOH : HOAC : H₂O (4:1:5) for 64 hours, air dried, and dipped through p-anisidine HCl reagent. The color intensity of sugar spots developed on the paper chromatogram was measured with a photovolt densitometer.
Table 2. Saccharides in the digests after incubating 30 mg raffinose with 30 mg sweet almond emulsin in 3 ml 0.2 M acetate buffer at pH 5, 35°C

<table>
<thead>
<tr>
<th>Saccharides</th>
<th>Incubation times (days)</th>
<th>raffinose</th>
<th>sucrose</th>
<th>fructose</th>
<th>galactose</th>
<th>glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>++</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Identified by paper chromatography.

Note: ++ present in high concentrations.
+ present in low concentrations.
- absent.
Table 3. Saccharides in the digests after incubating 30 mg raffinose with 30 mg sweet almond emulsin in 3 ml 0.2 M acetate buffer at pH 6, 35°C

<table>
<thead>
<tr>
<th>Incubation times (days)</th>
<th>Saccharides</th>
<th>raffinose</th>
<th>sucrose</th>
<th>fructose</th>
<th>galactose</th>
<th>glucose</th>
</tr>
</thead>
<tbody>
<tr>
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<td>++</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

a No sucrose hydrolysis occurred.

Note: ++ present in high concentrations.
  + present in low concentrations.
  - absent.
Table 4. Saccharides in the digests after incubating 30 mg raffinose with 30 mg sweet almond emulsin in 3 ml 0.2 M acetate buffer at pH 7, 35°C

<table>
<thead>
<tr>
<th>Incubation times (days)</th>
<th>raffinose</th>
<th>sucrose</th>
<th>fructose</th>
<th>galactose</th>
<th>glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>++</td>
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<td>-</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*a Partially hydrolyzed.

Note: ++ present in high concentrations.
+ present in low concentrations.
- absent.
raffinose was detectable in digests even after 4 days of incubation (96 hours). When the pH was raised to 8, no effects on raffinose were observed during four days of incubation.

The concomitant hydrolysis of sucrose during the above incubations was further explored using sucrose as the substrate under the conditions just cited. Table 5 is the results of the experiment. It reveals that the inversion of sucrose occurred in digests at pH 5 on the first day, but was delayed until the second day in pH 6 media and did not occur when the pH was raised to 7 during the four days of incubation. The same results as in pH 7 digests were noted when the incubation was carried out at pH 8. The above findings suggest the hydrolysis of sucrose in digests was due to the effects of acidic environment not the enzymatic action of emulsin since hydrolysis did not occur at pH 7 or 8. It also appears that if the raffinose in the sample is completely removed in one day of incubation at pH 6, the accompanying sucrose hydrolysis can be avoided by terminating the reaction at the end of one day.

When the enzyme was applied to molasses, the conditions were maintained the same as above except using 1.5 ml 5°Bx molasses containing 1.5 mg raffinose as substrate in a final volume of 3 ml. Table 6 shows the results of incubation at pH 5, 6, or 7. It is apparent raffinose in the molasses was completely eliminated at the end of one-day incubation at all three different pH conditions. At pH 5, an increase of glucose and fructose content in the reaction mixture was noted, which was apparently due to sucrose hydrolysis in an acidic medium. However, at pH 6, no sucrose hydrolysis in molasses was expected at the end of one-day incubation according to the above-mentioned
Table 5. Saccharides in the digests resulting from the incubation of 30 mg sucrose with 30 mg sweet almond emulsin in 3 ml 0.2 M acetate buffer at pH 5-7, 35°C

<table>
<thead>
<tr>
<th>Incubation time (days)</th>
<th>pH 5</th>
<th></th>
<th>pH 6</th>
<th></th>
<th>pH 7</th>
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</thead>
<tbody>
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<td></td>
<td>sucrose</td>
<td>fructose</td>
<td>glucose</td>
<td>sucrose</td>
<td>fructose</td>
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<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: ++ present in high concentrations. 
+ present in low concentrations. 
- absent.
Table 6. Saccharides in the digests after incubating 1.5 ml 5°B_{x} molasses solution containing 1.5 mg raffinose with 30 mg sweet almond emulsin in 1.5 ml 0.2 M acetate buffer at pH 5, 6, or 7, 35°C

<table>
<thead>
<tr>
<th>Saccharides</th>
<th>raffinose</th>
<th>sucrose</th>
<th>fructose</th>
<th>glucose</th>
<th>galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation times (days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
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</tbody>
</table>

Note: ++ present in high concentrations.
+ present in low concentrations.
- absent.
studies on sucrose inversion. The detectable amounts of glucose and fructose present in the reaction mixtures at pH 6 and 7 were believed to be the constituents of molasses, not the products of sucrose hydrolysis during the incubation. It was because same concentrations of both invert sugars were detected at pH 6 and 7 reaction mixtures by paper chromatographic method, while at pH 7 sucrose inversion should not take place. When pH was raised to 8 the raffinose in molasses solutions remained unaffected.

In summary, incubation of 30 mg commercial sweet almond emulsin (800 units/mg) with 2.5°Bx molasses which contains 1.5 mg raffinose in 3 ml 0.1 M acetate buffer at pH 5-7, 35°C for one day resulted in a complete hydrolysis of raffinose in the digest. To avoid undesirable sucrose hydrolysis and obtain a satisfactory speed, however, a medium acidity at pH 6 is recommended.

\(^1\)Change of concentration due to dilution.
SUMMARY

Incubation of 30 mg commercial sweet almond emulsin (800 units/mg) with 2.5°Bx molasses which contain 1.5 mg raffinose in 3 ml 0.1 M acetate buffer at pH 6, 35°C for one day resulted in a complete hydrolysis of raffinose in the digest. No sucrose inversion occurred under the above conditions.
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VITA

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Doctor of Philosophy

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