



Studies on application of Phosphoryl Oligosaccharides as functional food material

戸尾, 健二

(Degree)

博士 (農学)

(Date of Degree)

2004-09-17

(Date of Publication)

2010-08-24

(Resource Type)

doctoral thesis

(Report Number)

乙2777

(URL)

<https://hdl.handle.net/20.500.14094/D2002777>

※ 当コンテンツは神戸大学の学術成果です。無断複製・不正使用等を禁じます。著作権法で認められている範囲内で、適切にご利用ください。



神戸大学博士論文

**Studies on Application of Phosphoryl Oligosaccharides
as Functional Food Material**

(リン酸化オリゴ糖の機能性食品素材としての応用に関する研究)

平成 16 年 8 月

戸尾 健二

TABLE OF CONTENTS

INTRODUCTION	1
1. Phosphoryl oligosaccharides	1
2. Acid phosphatase	4
3. Problem of calcium absorption	5
4. Application of POs-Ca on dental health	8
5. Aim of this study	12
 CHAPTER I: Properties of a novel acid phosphatase in <i>Aspergillus</i>	
<i>niger</i> on separation of phosphoryl oligosaccharides ...	13
SECTION 1: Purification and characterization of a novel acid	
phosphatase in <i>Aspergillus niger</i> KU-8	13
1.1 Introduction	13
1.2 Materials and Methods	14
1.2.1 Material	14
1.2.2 Screening	14
1.2.3 High performance anion-exchange chromatography	
(HPAEC)	15
1.2.4 Identification of a selected fungus	15
1.2.5 Culture conditions and preparation of cell-free	
extract	15
1.2.6 Assay of acid phosphatase activity	15
1.2.7 Protein assay	16
1.2.8 Estimation of molecular weight by SDS-PAGE	16
1.2.9 Substrate specificity	17
1.2.10 Effects of various metal ions and chemical	
reagents	17
1.2.11 Action of ACPase II on PO-1	17
1.3 Results	19

1.3.1 Isolation of ACPase II-producing <i>Aspergillus niger</i> KU-8	19
1.3.2 Purification of ACPase II	19
1.3.3 Estimation of molecular weight	20
1.3.4 Effects of pH on activity and stability of ACPase II	22
1.3.5 Effects of temperature on activity and stability of ACPase II	23
1.3.6 Substrate specificity	24
1.3.7 Effects of various metal ions and chemicals	25
1.3.8 Action of ACPase II on PO-1	25
1.3.9 Properties of ACPase I	29
1.4 Discussion	31
1.5 Summary	34
SECTION 2: Substrate selectivity of acid phosphatase II in <i>Aspergillus niger</i> KU-8 on C-3 and C-6 phosphate groups of phosphoryl oligosaccharides	
2.1 Introduction	35
2.2 Materials and Methods	36
2.2.1 Enzymes	36
2.2.2 Other materials	36
2.2.3 Preparation of 3 ² -phosphoryl maltotriose and 6 ² -phosphoryl maltotriose	36
2.2.3.1 Fractionation of 3-phosphoryl oligosaccharides and 6-phosphoryl oligosaccharides by Daisopak ODS-B column chromatography	37
2.2.3.2 Preparation of 3 ² -phosphoryl maltotriose	37
2.2.3.3 Preparation of 6 ² -phosphoryl maltotriose	37
2.2.4 Assay of bacterial saccharifying α -amylase (BSA) activity	38
2.2.5 Assay of neopullulanase (NPL) activity	38

2.2.6 Assay of acid phosphate (ACPase) activity	38
2.2.7 Measurement of Michaelis constant (K_m) and molecular activity (k_{cat})	39
2.3 Results	40
2.3.1 Fractionation of 3-phosphoryl oligosaccharides and 6-phosphoryl oligosaccharides	40
2.3.2 Preparation of 3 ² -phosphoryl maltotriose	40
2.3.3 Preparation of 6 ² -phosphoryl maltotriose	40
2.3.4 Michaelis constant (K_m) and molecular activity (k_{cat}) ..	46
2.4 Discussion	49
2.5 Summary	51

CHAPTER II: Bioavailability of phosphoryl oligosaccharides of

calcium (POs-Ca) in rats..... 52

SECTION 1: *In vitro* solubility and *in vivo* digestibility of POs-Ca..... 52

1.1 Introduction	52
1.2 Materials and Methods.....	53
1.2.1 Materials	53
1.2.2 Preparation of POs and POs-Ca	53
1.2.3 Comparison of <i>in vitro</i> calcium solubility	54
1.2.4 Digestibility of POs-Ca by artificial gastric acid	54
1.2.5 Effect of POs-Ca administration on plasma glucose response in rats.....	54
1.2.5.1 Animals	54
1.2.5.2 Administration of glucose, high maltotetraose syrup or POs-Ca to rats	55
1.2.6 Measurement of plasma glucose, calcium and phosphate concentration.....	55
1.2.7 Statistical analysis	55
1.3 Results	56

1.3.1 Comparison of calcium solubility	56
1.3.2 Digestibility of POs-Ca by artificial gastric acid	58
1.3.3 Effect of POs-Ca administration on plasma glucose response in rats.....	59
1.3.4 Effect of POs-Ca administration on plasma calcium and phosphate in rats.....	61
1.4 Discussion.....	62
1.5 Summary	64
SECTION 2: Calcium absorbability of POs-Ca in comparison with various calcium compounds and effect of POs-Ca feeding in rats.....	
2.1 Introduction	65
2.2 Materials and Methods.....	66
2.2.1 Chemicals	66
2.2.2 Comparison of <i>in situ</i> calcium absorbability using rat ligated jejunum loop	66
2.2.2.1 Animals	66
2.2.2.2 Time-course characteristics of calcium absorption from POs-Ca	66
2.2.2.3 Comparison of calcium absorbability.....	68
2.2.3 Comparison of calcium absorbability with the combined use of a whey mineral complex (WMC) and POs-Ca using rat ligated jejunum loop	69
2.2.4 Effect of POs-Ca on rat growth and calcium absorption	70
2.2.4.1 Animals and diets.....	70
2.2.4.2 Calcium balance study	71
2.2.4.3 Analysis of serum.....	71
2.2.4.4 Analysis of feces and urine.....	71
2.2.5 Statistical analysis	72

2.3 Results	73
2.3.1 Comparison of <i>in situ</i> calcium absorbability	73
2.3.1.1 Time-course characteristics of calcium absorption from POs-Ca	73
2.3.1.2 Comparison of calcium absorbability from POs-Ca with various calcium compounds.....	75
2.3.2 Comparison of calcium absorbability with the combined use of a whey mineral complex (WMC) and POs-Ca	76
2.3.3 Effect of POs-Ca on rat growth and calcium absorption	78
2.3.3.1 Body weight and food intake, liver and cecum weight	78
2.3.3.2 Serum analysis	78
2.3.3.3 Calcium balance.....	79
2.4 Discussion.....	80
2.5 Summary	84

CHAPTER III: Remineralization effect of chewing gum containing phosphoryl oligosaccharides of calcium (POs-Ca) on enamel lesion in children <i>in vitro</i>.....	85
1. Introduction	85
2. Materials and Methods	86
2.1 Subjects.....	86
2.2 Test gums.....	86
2.3 Preparation of demineralized bovine enamel slabs.....	86
2.4 <i>In vitro</i> remineralization test by collected saliva.....	86
2.5 Quantification of mineral distribution	87
2.6 Measurement of salivary volume, pH, calcium and phosphate.....	90

2.7 Statistical analysis	90
3. Results	91
3.1 Comparison of properties of the stimulated whole saliva	91
3.2 Mineral distributions	92
4. Discussion	94
5. Summary	100
CONCLUSION.....	101
1. Characterization of acid phosphatase to understand the function of POs	101
2. Application of POs-Ca as soluble calcium material	102
3. Application of POs-Ca as remineralization enhancer	103
4. Further works.....	104
ACKNOWLEDGEMENTS.....	108
REFERENCES.....	109
LIST OF PUBLICATIONS.....	126

ABBREVIATIONS

ACPase	acid phosphatase
BSA	bacterial saccharifying α -amylase
DP	degree of polymerization
ΔZ	mineral loss value
F6P	D-fructose 6-phosphate
G1P	D-glucose 1-phosphate
G6P	D-glucose 6-phosphate
HPAEC	high-performance anion-exchange chromatography
HPLC	high-performance liquid chromatography
HSI test	human saliva immersing test
ICP	inductively coupled plasma atomic emission spectrometry
ld	lesion depth
NPL	neopullulanase
PAD	pulsed amperometric detector
pNPP	<i>p</i> -nitrophenyl phosphate
POs	phosphoryl oligosaccharides
POs-Ca	phosphoryl oligosaccharides of calcium
PO-1	phosphoryl oligosaccharides-1 fractionated from POs
PO-2	phosphoryl oligosaccharides-2 fractionated from POs
TETRUP-H	high-maltotetraose syrup (a trade mark)
TLC	thin-layer chromatography
WMC	whey mineral complex

3 ² -phosphoryl maltotriose	<i>O</i> -α-D-glucopyranosyl-(1→4)-(3- <i>O</i> -phosphoryl)- <i>O</i> -α-D-glucopyranosyl-(1→4)-D-glucose
3 ³ -phosphoryl maltotetraose	<i>O</i> -α-D-glucopyranosyl-(1→4)-(3- <i>O</i> -phosphoryl)- <i>O</i> -α-D-glucopyranosyl-(1→4)- <i>O</i> -α-D- glucopyranosyl-(1→4)-D-glucose
3 ⁴ -phosphoryl maltopentaose	<i>O</i> -α-D-glucopyranosyl-(1→4)-(3- <i>O</i> -phosphoryl)- <i>O</i> -α-D-glucopyranosyl-(1→4)- <i>O</i> -α-D- glucopyranosyl-(1→4)- <i>O</i> -α-D-glucopyranosyl- (1→4)-D-glucose
6 ² -phosphoryl maltose	6- <i>O</i> -phosphoryl- <i>O</i> -α-D-glucopyranosyl-(1→4)-D- glucose
6 ² -phosphoryl maltotriose	<i>O</i> -α-D-glucopyranosyl-(1→4)-(6- <i>O</i> -phosphoryl)- <i>O</i> -α-D-glucopyranosyl-(1→4)-D-glucose
6 ³ -phosphoryl maltotriose	6- <i>O</i> -phosphoryl- <i>O</i> -α-D-glucopyranosyl-(1→4)- <i>O</i> - α-D-glucopyranosyl-(1→4)-D-glucose
6 ³ -phosphoryl maltotetraose	<i>O</i> -α-D-glucopyranosyl-(1→4)-(6- <i>O</i> -phosphoryl)- <i>O</i> -α-D-glucopyranosyl-(1→4)- <i>O</i> -α-D- glucopyranosyl-(1→4)-D-glucose
6 ⁴ -phosphoryl maltopentaose	<i>O</i> -α-D-glucopyranosyl-(1→4)-(6- <i>O</i> -phosphoryl)- <i>O</i> -α-D-glucopyranosyl-(1→4)- <i>O</i> -α-D- glucopyranosyl-(1→4)- <i>O</i> -α-D-glucopyranosyl- (1→4)-D-glucose

INTRODUCTION

1. Phosphoryl oligosaccharides

Potato starch contains esterified phosphate groups, and the ester phosphate groups are found exclusively in amylopectin in its components (Schoch *et al.*, 1942, Gracza, 1965). Hizukuri and his co-workers quantitatively determined the phosphate at C-6 position of glucose residue and observed the presence of unknown phosphate (Hizukuri *et al.*, 1970), later identified as glucose 3-phosphate (Tabata *et al.*, 1971), and also suggested the presence of trace amounts of glucose 2-phosphate. Approximately 60% to 70% of the phosphate groups were bound to C-6 position of glucosyl residues almost all the rest being at C-3 (Takeda *et al.*, 1981). Takeda and Hizukuri (1982) reported that the phosphate groups were located mostly in the B-chain of amylopectin, whereas there was very little phosphorylation of amylose. Suzuki *et al.* (1994) reported that potato amylopectin contains 200-1,000 ppm of the ester phosphate. Incorporation of phosphate into starch has been suggested to be parallel with starch synthesis in potato (Nielsen *et al.*, 1994).

The potato use in Japan in 2001 is shown in Table 1. Of the total potato yield in one year, 38% (about 1,140,000 tons) were cultivated for the purpose of starch materials, and about 230,000 tons of potato starches were manufactured in Japan. Approximately 50% of potato starch is used for production of glucose, high-fructose syrup or dextrin in starch-saccharifying industry. The rest are used mainly for fish paste products and other processed foods.

Table 1. Potato Use in Japan in 2001

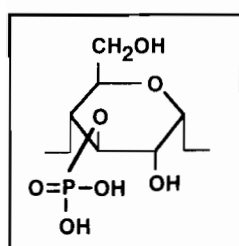
Purpose	%
Starch	38.6
Edible	22.0
Processed food	17.1
Self-use by farmers	8.8
Seed	6.1
Feed	0.7
Others	6.7

Data are referred from the report of The Ministry of Agriculture, Forestry and Fisheries of Japan.

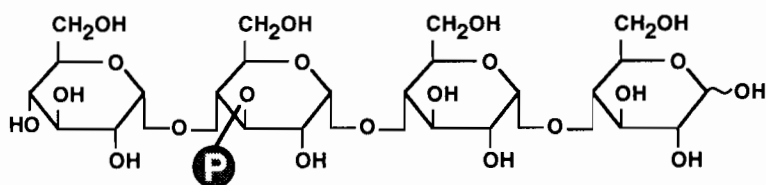
Phosphoryl oligosaccharides (POs) are enzymatic hydrolysates of potato starch and the yield from potato starch is about 1% (Kamasaka *et al.*, 1997b). Kamasaka *et al.* (1995) noted the method of production of POs from potato starch by using bacterial liquefying α -amylase (EC 3.2.1.1), glucoamylase (EC 3.2.1.3) and pullulanase (EC 3.2.1.41), and newly found the inhibitory effect of the POs on the formation of calcium-phosphate precipitate. The actions of these amylolytic enzymes are hindered by the phosphate groups linked to the glucosyl residues of amylopectin, hence POs are obtained as indigestible components by the enzymes. Furthermore, it turned out that POs were contained in the waste water generated in the industrial saccharifying process of potato starch so that POs could be obtained on an industrial scale.

POs are separated into major and minor fractions, PO-1 and PO-2, by the number of the phosphate groups linked to each molecule. The average degree of polymerization of dephosphorylated PO-1 and PO-2 fraction is evaluated to be 4.02 and 5.82, respectively.

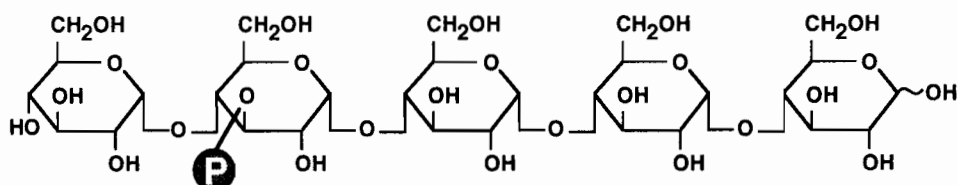
The detailed structures of PO-1 fraction were analyzed by Kamasaka *et al.* (1997a), as shown in Fig. 1. PO-1 fraction is made up of 3-phosphoryl



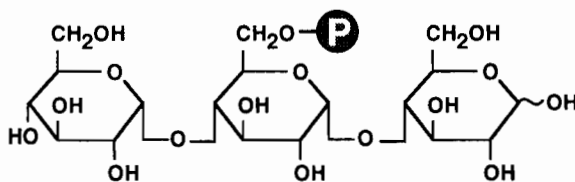
C-3 bound
phosphate
group



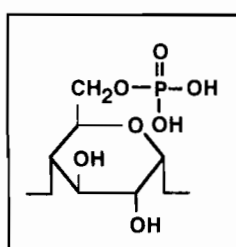
3³-phosphoryl maltotetraose



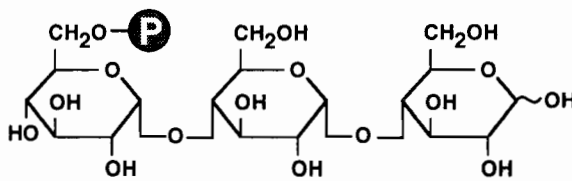
3⁴-phosphoryl maltopentaose



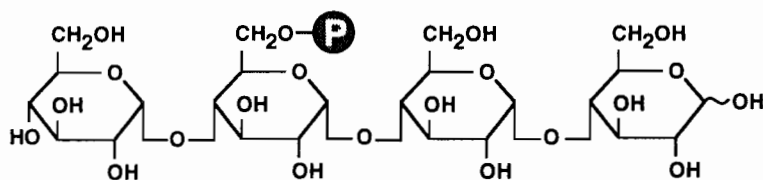
6²-phosphoryl maltotriose



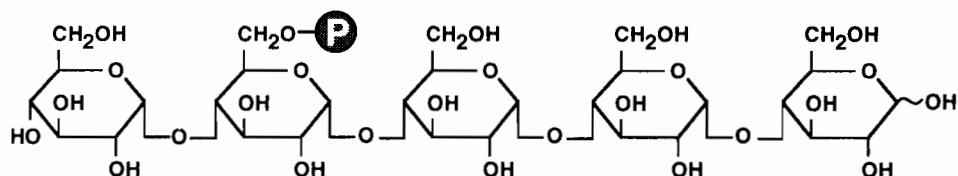
C-6 bound
phosphate
group



6³-phosphoryl maltotriose



6³-phosphoryl maltotetraose



6⁴-phosphoryl maltopentaose

Fig. 1. The Structures of POs in PO-1 Fraction.

P ; phosphate group

oligosaccharides (3³-phosphoryl maltotetraose and 3⁴-phosphoryl maltopentaose) and 6-phosphoryl oligosaccharides (6³-phosphoryl maltotetraose, 6²-phosphoryl maltotetraose, 6³-phosphoryl maltotetraose and 6⁴-phosphoryl maltopentaose). The binding ratio of the phosphate groups at C-3 and C-6 is approximately 3:7 in PO-1 fraction. PO-2 fraction is predominantly composed of maltopentaose and maltohexaose to which at least two phosphate groups were attached (Kamasaka *et al.*, 1997d).

2. Acid phosphatase

In biological systems, hydrolysis of phosphate monoester is an important reaction for energy metabolism, metabolic reaction and signal transduction pathways. Enzymatic hydrolysis of phosphate monoester is performed by phosphatases, which are five well-characterized classes of phosphatase. They are often referred to as acid or alkaline phosphatases based on whether their optimum pH for hydrolysis is below or above pH 7; (1) alkaline phosphatases, (2) purple acid phosphatases, (3) low molecular weight acid phosphatases, (4) high molecular weight acid phosphatases, and (5) protein phosphatases that are specific for phosphoserine or phosphothreonine residues (Vincent *et al.*, 1992, Kostrewa *et al.*, 1999).

Acid phosphatases (orthophosphoric monoester phosphohydrolases, EC 3.1.3.2) hydrolyze a wide variety of phosphate monoester. The enzymes are widely distributed in nature and occur in a variety of organs and tissues (Hollander, 1971).

There have been many reports on the general substrate specificity of acid phosphatases and phytases, a kind of acid phosphatases hydrolyzing phytic acid (Irving and Cosgrove, 1974, Ullah and Cummins, 1987 and 1988, Ullah and Phillippy, 1994, Wodzinski and Ullah, 1996, Wyss *et al.*, 1999a, 1999b).

However, there have been no reports that have described the specificity

toward the different binding position of phosphate groups of phosphorylated saccharides such as a PO-1 fraction of POs. In addition, there are at present no commercially available standard samples of C-3 phosphoryl saccharide, and C-3 phosphate groups of PO-1 fraction are hydrolyzed under acidic condition that C-6 phosphate groups are not.

Therefore, it would be useful that the study of phosphatase which has high substrate specificity toward the binding position of C-3 or C-6 phosphate groups of POs in that enzymatic preparation of standard samples, especially 3-phosphoryl oligosaccharides, on a large scale. The study will lead to better understanding of the relationship between the difference of the binding position of phosphate groups of POs and the difference of the function of each POs.

3. Problem of calcium absorption

The adult human body contains about 1-1.2 kg of calcium, which amounts to about 1.5-2% of body weight. Of this, 99% is found in mineralized tissues, such as bones and teeth, where it is present as calcium phosphate (together with a small component of calcium carbonate), providing rigidity and structure (Cashman, 2002, Nordin, 1997). The remaining 1%, found in blood, extracellular fluid, muscle, and other tissues, play an important role in mediating vascular contraction and vasodilation, muscle contraction, nerve transmission and glandular secretion (Institute of Medicine, 1997).

Calcium is required for natural growth and development of the skelton (National Research Council, 1989, Nordin, 1997). Calcium requirements vary throughout an individual's life, with greater needs during the periods of rapid growth in childhood and adolescence, during pregnancy and lactation, and in later life.

According to National Nutrition Survey in Japan in 2002, the average

amount of calcium intake in Japanese people was 546 mg/day. Large population of Japanese, especially 20-29 yr, failed to achieve the dietary allowance of calcium (Table 2). Chronic calcium deficiency resulting from inadequate intake or poor intestinal absorption causes a wide variety of such disease as osteoporosis, hypertension, and cancer.

Table 2. Calcium Intake and Sufficiency in Japanese in 2002

Male				
Age (yr)	Number	Intake (mg/day)	Allowance (mg/day)	Sufficiency (I/A, %)
1-6	346	517	500-600	86-103
7-14	452	770	600-900	86-128
15-19	301	621	700-800	78-89
20-29	540	471	700	67
30-39	657	475	600	79
40-49	677	507	600	85
50-59	891	533	600	89
60-69	801	613	600	102
>70	712	582	600	97

Female				
Age (yr)	Number	Intake (mg/day)	Allowance (mg/day)*	Sufficiency (I/A, %)
1-6	341	481	500-600	80-96
7-14	464	671	600-700	96-112
15-19	314	487	600-700	71-80
20-29	608	458	600	76
30-39	723	475	600	79
40-49	743	500	600	83
50-59	996	560	600	93
60-69	885	598	600	100
>70	1,040	539	600	90

*Pregnancy; +300, Lactation; +500.

Data are referred from the report of Ministry of Health, Labour and Welfare.

It is well-known the fact that at least some of the ingested calcium will be dissolved in stomach acid. However, absorption of calcium does not take place from the stomach but from the small intestine where pH is commonly above 6.5 (Heaney *et al.*, 1990). Duflos *et al.* (1995) and Bronner *et al.* (1999) also showed the pH in the all segments of the rat intestine being above 6.6. Although the relationship between the solubility and bioavailability of calcium remains unclear (Heaney *et al.*, 1990), it is obvious that calcium must be ionized and in solution to be absorbed (Bronner *et al.*, 1986, Bronner, 1988, Pansu *et al.*, 1989). It is widely recognized that phosphate salt tends to form an insoluble complex with calcium under such intestinal conditions (Naito, 1986). It is important to prevent the formation of insoluble calcium-phosphate complex and to keep the solubility of calcium high in the intestine.

A number of food constituents have been suggested as potential enhancers of calcium absorption. For example, phosphopeptide derived from milk casein (casein phosphopeptide; CPP) has been proposed as potential enhancers of calcium absorption (Sato *et al.*, 1986, Lee *et al.*, 1992, Saito *et al.*, 1998). Berrocal *et al.* (1989) demonstrated that such phosphopeptide have the capacity to chelate calcium and to prevent the precipitation of calcium phosphate salts and suggested that they may help to maintain a high concentration of soluble calcium in the intestinal lumen.

The phosphate groups attached to phosphoryl oligosaccharides are able to bind calcium as well as CPP, and POs have an inhibitory effect of the formation of calcium-phosphate precipitate as mentioned above. Thus the application of POs in the food industry as an enhancer of calcium absorption or calcium supplement in the form of calcium salt (phosphoryl oligosaccharides of calcium; POs-Ca) would be expected.

4. Application of POs on dental health

Dental decay and subsequent loss of teeth was believed to be inevitable. Much has been learned about the process of dental caries, and measures have been introduced to slow or even arrest progress during the 20th century. Early caries lesions, when diagnosed, have traditionally been treated by excavation and the placement of metallic or synthetic restorations.

A most exciting finding in recent years has been discovered that the outer enamel surface of the tooth is constant process of dissolving (demineralization) and reforming (remineralization). Figure 2 shows the structure of tooth.

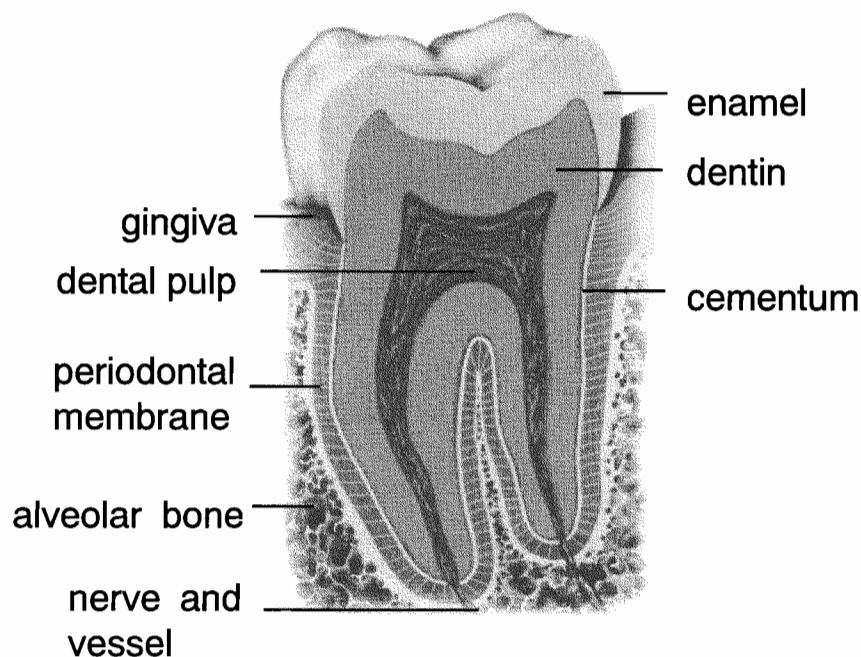
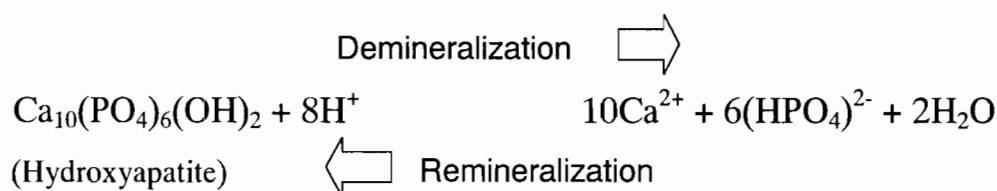


Fig. 2. The Structure of Tooth.

Enamel caries occur as a result of a shift in equilibrium between the demineralization and remineralization processes, with demineralization predominating. The caries process is initiated by the dissolution of tooth mineral by organic acids produced by the action of plaque bacteria, mainly mutans streptococci and lactobacilli, on fermentable carbohydrates. When demineralization predominates, the initial caries lesion progress to cavitation. Remineralization can arrest the caries process, thus avoiding the need for restoration.

The chemistry involved in demineralization and remineralization of enamel is shown below (Kashket, 1999).



It is apparent that the addition of acid (H^+) would shift the equilibrium toward demineralization, as happens during a cariogenic challenge. The addition of calcium ions and/or phosphate ions would favor remineralization, even though the environment might be mildly acidic.

It is now recognized that saliva is important for oral health. The oral cavity and exposed tooth surfaces are continuously bathed in saliva. Saliva serves many different functions in promoting oral health including protection against dental caries. Saliva is secreted from the major and minor salivary glands and the secretory components of these glands can be divided into two major categories; (1) water and electrolytes and (2) macromolecules, particularly proteins and glycoproteins.

A study of saliva and its tooth-protective components reveals at least four important functions of saliva; (1) buffering ability, (2) a cleansing effect, (3) antibacterial action, and (4) maintenance of a saliva

supersaturated in calcium phosphate (Dowd, 1999). It is one of the most important factors in preventing and/or reducing dental caries, and in maintaining a neutral pH in plaque and in the oral cavity (Itthagarun, 1997).

It is now well established that chewing sugar-free gum immediately after a carbohydrate-induced pH fall in plaque leads to a rapid and sustained rise in pH (Manning and Edgar, 1993), presumably due in the main to the increased buffering ability of stimulated saliva. Of its important characteristics on dental health, chewing gum stimulates the salivary flow rate at levels about 3 to 10 times resting values. The stimulation of salivary flow leads to an increase in its caries-protective properties, notably its ability to buffer acids in plaque formed from dietary carbohydrate, and its increased salivary supersaturation with respect to calcium and phosphate leading to enhanced remineralization of early stage of caries (Edgar *et al.*, 1994, Edgar, 1998).

During the last two decades, considerable progress has been made in the understanding of dental caries. Transversal (or contact) microradiography has been conventionally applied to visualize mineral distribution in caries lesions in enamel in mainly histopathological studies. Arends and co-workers established mineral parameters that quantify microradiographically determined mineral distribution, for example, the lesion depth (ld, μm) and the mineral loss value (ΔZ , $\text{vol}\% \cdot \mu\text{m}$) (Arends *et al.*, 1989 and 1990, Arends and ten Bosch, 1992). These parameters are now widely accepted as standard mineral parameters in caries research.

Inaba *et al.* (1997a, 1997b) newly advocated an original computer-assisted measurement program for microradiographical mineral distribution parameters; mineral distribution analysis (MDA) program combined with computer-associated videodensitometry. Due to the

simple operation procedures, high reproducibility and comparison of standardized basis mineral parameter values, the MDA program is a useful tool in caries research.

Recently, Inaba *et al.* (2002a, 2002b, 2002c, 2003) and Kamasaka *et al.* (2002) demonstrated that the significant remineralization effect of sugar-free chewing gum containing 2.5% POs-Ca (POs-Ca (+) gum) on artificial early caries lesions in enamel *in vitro* and *in situ*. The possible mechanism for enhanced remineralization in the POs-Ca (+) gum is demonstrated by Inaba *et al.* (2002a) as follows; (1) stimulation of salivary flow by mastication of POs-Ca (+) gum, (2) increase in salivary pH by increased salivary flow and (3) increase in salivary soluble calcium concentration by release from POs-Ca and in molar ratio of salivary calcium/phosphate to the near rate of hydroxyapatite (≈ 1.67), resulting in remineralization.

However, all data of remineralization effect by POs-Ca (+) gum were obtained with the adult volunteers. There was no data indicating the effectiveness of POs-Ca (+) gum to children. Caries prevention and enhancement of remineralization is important not only for adults but also for children, because children have more opportunities for intake of fermentable saccharides such as sucrose and glucose from daily meals or snacks.

With respect to the remineralization study, Kamasaka *et al.* (2002) newly demonstrated the *in vitro* human saliva immersing (HSI) test. The result of HSI-test corresponds to that of *in situ* intraoral study (Inaba *et al.*, 2002a, Kamasaka *et al.*, 2002). Furthermore, the HSI-test makes it easy to standardize the experimental conditions and is easier task for volunteers than *in situ* intraoral study. It is therefore thought that HSI-test could be an alternative method for the intraoral study and also particularly useful in examination by children volunteers.

5. Aim of this study

It became clear that the POs were contained in the waste water generated in the industrial saccharifying process of potato starch so that POs could be obtained on an industrial level.

The aim of this study was to characterize a novel acid phosphatase from *Aspergillus niger* specific to the C-6 phosphate groups of POs for the purpose of enzymatic preparation of 3-phosphoryl oligosaccharides from POs, and to investigate the bioavailability of phosphoryl oligosaccharides of calcium (POs-Ca) and the remineralization effect of POs-Ca on early enamel lesion.

This thesis deals with a novel property of a fungal acid phosphatase for phosphate ester groups of POs, with bioavailability of POs-Ca as a calcium material for foods and with application of POs-Ca to chewing gum as a remineralization enhancer.

In Chapter I, the author described the characterization of an acid phosphatase purified from newly isolated *Aspergillus niger* KU-8 and its novel substrate specificity toward the C-3 and C-6 phosphate groups of POs.

In Chapter II, the author described the bioavailability of POs-Ca, namely, *in vivo* digestibility as a new oligosaccharide, *in situ* absorbability as a new calcium source.

In Chapter III, the author described the remineralization effect on enamel lesion by sugar-free chewing gum containing POs-Ca in children by *in vivo* HSI test.

CHAPTER I

Properties of a novel acid phosphatase in *Aspergillus niger* on separation of phosphoryl oligosaccharides

SECTION 1

Purification and characterization of a novel acid phosphatase in *Aspergillus niger* KU-8

1.1 Introduction

Potato amylopectin is known to contain a small amount of covalently-bound phosphate groups. Of those, approximately 70% were bound at C-6 position of glucosyl residue, and almost all the rest at C-3. Phosphoryl oligosaccharides (POs) have phosphate groups as the same binding ratio of C-3 to C-6 as amylopectin. POs are fractionated into two fractions, PO-1 and PO-2. PO-1 fraction is composed of 3-phosphoryl oligosaccharides and 6-phosphoryl oligosaccharides having one phosphate group in each molecule. Little is known of the property of C-3 phosphate groups because there are at present no commercially available standard samples of C-3 phosphoryl saccharides, although those of C-6 phosphoryl saccharides such as glucose 6-phosphate are available.

Acid phosphatases (EC 3.1.3.2) are widely distributed in nature and occur in a variety of organisms and tissues. Although several studies on acid phosphatases from *Aspergillus niger* have been reported (Shimada *et al.*, 1974, 1977, Komano, 1975, Zyla, 1980), there have been no reports that have described the different specificity toward C-6 and C-3 phosphate groups of phosphoryl saccharides such as PO-1.

By using PO-1 as a substrate, the author succeeded in isolating a fungus,

Aspergillus niger KU-8, producing two types (I and II) of intracellular acid phosphatases which differ in some properties.

In this section, the author described the purification and characterization of a novel intracellular acid phosphatase, ACPase II, from *A. niger* KU-8.

1.2 Materials and Methods

1.2.1 Materials

Phenyl Sepharose, Q-Sepharose, and Superdex G-200 were purchased from Pharmacia Fine Chemicals Co. TSKgel SuperQ-5PW was purchased from Tosoh Co. CarboPac PA-100 (0.4 ϕ x 25 cm) was purchased from Dionex Corp. (Sunnyvale, CA, U.S.A.). Standard maltooligosaccharides with degrees of polymerization of 2 to 6 were purchased from Sigma Chemicals, Hayashibara Biochemical Lab., and Funakoshi Pharmaceutical Co., respectively. Fructose 6-phosphate and *p*-nitrophenyl phosphate (pNPP) were purchased from Wako Pure Chemical Industries. Glucose 6-phosphate (G6P) and sodium phytic acid were purchased from Nacalai Tesque. Glucose 1-phosphate (G1P) and fructose 1,6-bisphosphate were purchased from Boehringer Mannheim GmbH and Fluka BioChemika, respectively. All other chemicals and materials used were of analytical or commercial grade. PO-1 was prepared from potato starch hydrolysate according to the method of Kamasaka *et al.* (1995).

1.2.2 Screening

Screening of phosphatase-producing microorganisms from soil was done on plates of a medium (pH 6.3) containing 0.5% glucose, 0.1% yeast extract (Difco Laboratories), 0.5% Polypepton (Nihon Seiyaku), 0.1% NaCl, 0.1% KCl, 0.02% MgSO₄ · 7H₂O, 0.002% FeSO₄ · 7H₂O, 0.1% polyphosphate (Wako Pure Chemical Industries), and 2% agar.

Microorganisms that had showed good growth at 37°C on the plate were selected. Selected organisms were cultured on the liquid medium at 37°C for 1 to 3 days. Their phosphatase activities of both culture broth and cell-free extract were spectrophotometrically assayed using pNPP as a substrate, and the actions of the phosphatases on PO-1 were analyzed with high performance anion-exchange chromatography (HPAEC).

1.2.3 High performance anion-exchange chromatography (HPAEC)

HPAEC analysis of fraction PO-1 was done with a Dionex DX-300 gradient chromatography system under the following conditions: column, CarboPac PA-100; detection, pulsed amperometric detector (PAD); meter scale, 1 µC; temperature, ambient; flow rate, 1 mL/min. Elution was done by 0.1 M NaOH containing the following gradient of 1 M sodium acetate; 0% at 0 min, 10% at 12 min, 20% at 32 min, 20% at 37 min and 80% at 57 min.

1.2.4 Identification of a selected fungus

Morphological and cultural characteristics of a selected fungus and production of mycotoxins were examined by Japan Food Research Laboratories.

1.2.5 Culture conditions and preparation of cell-free extract

Aspergillus niger KU-8 was cultured in 2-liter shaking flask with 500 mL of the medium at 37°C for 4 days with agitative shaking. After cultivation, the mycelia collected by filtration was suspended in 10 mM acetate buffer (pH 4.5), and disrupted by sonic oscillation at 4°C for 60 min at 200 W. After centrifugation of the disrupted mycelial homogenate, the supernatant was used as a cell-free extract.

1.2.6 Assay of acid phosphatase activity

Acid phosphatase activity was measured by spectrophotometric method using pNPP or G6P as a substrate.

(1) pNPP: The reaction mixture containing 0.5 mL enzyme solution (at various concentrations), 1 mL of 0.2 M acetate buffer (pH 4.5) and 0.5 mL of 20 mM pNPP solution were incubated at 37°C for 15 min. The reaction was stopped by addition of 2 mL of 0.2 M Na₂CO₃, and the *p*-nitrophenol released was measured by the absorbance at 400 nm. One unit of activity was defined as the amount of enzyme which releases 1 µmol of *p*-nitrophenol per min under the assay conditions.

(2) G6P: The concentration of inorganic phosphate was measured by a modification of the method of Itaya and Ui (1966). The reaction mixture, containing 125 µL enzyme solution (at various concentrations), 250 µL of 0.2 M acetate buffer (pH 4.5) or glycine-HCl buffer (pH 2.0), and 125 µL of 20 mM G6P solution was incubated at 37°C for 15 min. Then 50 µL of 10 N perchloric acid, 150 µL of 20 mM ammonium vanadate containing 0.04 N perchloric acid, and 300 µL of 3.53% ammonium molybdate were added to the reaction mixture, and the total mixture was left for 30 min at room temperature. The absorbance at 420 nm was measured. One unit of activity was defined as the amount of enzyme which releases 1 µmol of inorganic phosphate per min under the assay conditions.

1.2.7 Protein assay

Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as a standard. During the enzyme purification with column chromatography, the protein elution profile was monitored spectrophotometrically as the absorbance at 280 nm.

1.2.8 Estimation of molecular weight by SDS-PAGE

SDS-PAGE by the Laemmli system (1970) was used for confirmation of the purification and for estimation of the molecular weight. Enzyme

solution was put on a precast SDS 8-16% gradient gel (TEF corporation), and run at 20 mA for 90 min. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250. A molecular weight marker kit (Bio-Rad Laboratories) was used as standard proteins.

1.2.9 Substrate specificity

The reaction mixture, containing 125 μ L of 20 mM substrate, 125 μ L of purified enzyme solution (at various concentrations) and 250 μ L of 0.2 M glycine-HCl buffer (pH 2.0) was incubated at 37°C for 15 min, and the released inorganic phosphate was measured by the method described above. The rate of hydrolysis was expressed against that of pNPP as 100%.

1.2.10 Effects of various metal ions and chemical reagents

The reaction mixture, containing 50 μ L of purified enzyme solution, 25 μ L of various metal ions or chemical reagents (final conc. 0.1-10 mM) and 300 μ L of 0.2 M glycine-HCl buffer (pH 2.0) was incubated at 37°C for 15 min. Then 125 μ L of 20 mM G6P solution was added and incubated at 37°C for 15 min, and the released inorganic phosphate was measured by the method described above. The relative activity was expressed as a percentage of the enzyme activity in the absence of metal ions or chemical reagents.

1.2.11 Action of ACPase II on PO-1

To analyze the action pattern of ACPase II on PO-1, 10 μ L of 30 mg/mL PO-1 was incubated with 10 μ L of 10 U/mL ACPase II solution and 10 μ L of 0.2 M glycine-HCl buffer (pH 2.0) at 37°C for 5 h. After the reaction was stopped by removing the enzyme with ultrafiltration with Ultrafree-MC (nominal molecular weight limit of 30,000, Millipore), PO-1 left in the reaction mixture was analyzed with HPAEC. For the analysis of PO-1, 4 μ L of sample was mixed with 36 μ L of distilled water and 25 μ L

was applied to HPAEC. To analyze the course of the reaction with PO-1, 100 μ L of 30 mg/mL PO-1 was incubated with 100 μ L of 10 U/mL enzyme solution and 100 μ L of 0.2 M glycine-HCl buffer (pH 2.0) at 37°C. At intervals, 30 μ L of the reaction mixture was removed, the reaction was stopped by ultrafiltration, and PO-1 left in the reaction mixture was analyzed with HPAEC.

1.3 Results

1.3.1 Isolation of ACPase-producing *Aspergillus niger* KU-8

Approximately 200 strains isolated from soil were aerobically cultured at 37°C for 1 to 3 days in a medium as described in materials and methods. After fractionation of cells and culture broth, phosphatase (acid and alkaline) activities of both cell-free extract and culture broth were assayed by using pNPP as a substrate. Strains having sufficient phosphatase activity were selected, and then the enzyme action on PO-1 was analyzed with HPAEC. After all, one of the 200 strains, KU-8, was isolated as a producer of an acid phosphatase of interest. The strain KU-8 was identified as *A. niger*, and mycotoxins were not detected in the culture broth at all.

1.3.2 Purification of ACPase II

ACPase II was purified from the cell-free extract of *A. niger* KU-8 as follows. Ammonium sulfate was added to the cell-free extract to 80% saturation and the solution was left overnight. The suspension was centrifuged at 40,000 *g* for 30 min. The supernatant, which contained almost all of the enzyme activity, was put on a Phenyl Sepharose column (2.6 ϕ x 10 cm) equilibrated with 80% saturation of ammonium sulfate in 10 mM acetate buffer (pH 4.5). ACPases (I and II) were co-eluted with a stepwise at a flow rate of 3 mL/min. The active fraction was dialyzed against 10 mM acetate buffer (pH 4.5). The dialyzed solution was put on a Q-Sepharose column (1 ϕ x 10 cm) equilibrated with the same buffer and then eluted with a 0 to 0.2 M NaCl gradient at a flow rate of 1 mL/min. At this stage, ACPases I and II were separated, and the fractions containing ACPase II were collected and dialyzed against 10 mM acetate buffer (pH 4.5). ACPase I showed weak reaction and no specificity for PO-1 in spite of its much higher activity for pNPP than ACPase II. The dialyzed

solution was separated on a TSKgel SuperQ-5PW column (0.8 ϕ x 7.5 cm) equilibrated with the same buffer and then eluted with a 0 to 0.2 M NaCl gradient at a flow rate of 0.5 mL/min. The fraction of ACPase II was concentrated by ultrafiltration with Centriprep 30 (Amicon division, W. R. Grace & Co.) and separated on a Superdex G-200 column (1.6 ϕ x 60 cm) equilibrated with the same buffer containing 0.1 M NaCl and eluted at a flow rate of 0.5 mL/min. The purification steps are summarized in Table 1. The final enzyme preparation was purified about 16-fold the cell-free extract, the yield being 5.6%. The enzyme activity at each step was measured using pNPP as a substrate.

Table 1. Summary of Purification of ACPase II from *A. niger* KU-8

Step	Total unit	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold
Cell extract	1112	100.00	11.1	100.0	1.0
80% satu. (NH ₄) ₂ SO ₄ sup.	1028	-	-	92.4	-
Phenyl Sepharose	1232	5.25	234.7	110.8	21.1
Q-Sepharose	168	1.86	90.3	15.1	8.1*
TSKgel SuperQ-5PW	100	0.76	131.6	9.0	11.8
Superdex G-200	62	0.34	182.4	5.6	16.4

*ACPase I and II were separated at this stage.

1.3.3 Estimation of molecular weight

The molecular weight of the purified ACPase II was estimated to be 66 kDa by SDS-PAGE (Fig. 1) and approximately 260 kDa by gel filtration on TSKgel G2000SW_{XL} (data not shown).

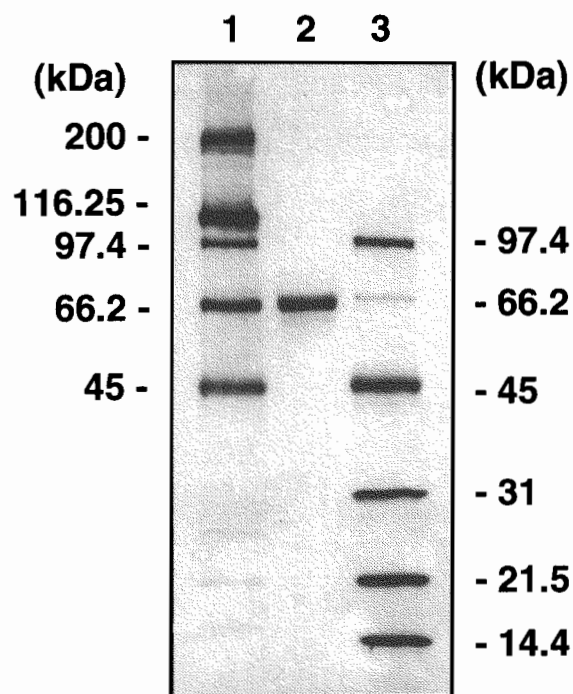


Fig. 1. SDS-PAGE of Purified ACPase II.

Lanes 1 and 3, molecular weight markers [myosin (200 kDa), *E. coli* β -galactosidase (116.25 kDa), rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white albumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa)]; lane 2, purified ACPase II.

1.3.4 Effects of pH on activity and stability of ACPase II

The effects of pH on activity and stability of ACPase II are shown in Fig. 2. The enzyme activity was measured using G6P as a substrate, because G6P was more stable than pNPP over a wide pH range. The optimum pH for the enzyme activity was 2.0 (Fig. 2a), and same results were also seen when pNPP was used as a substrate. ACPase II was stable from pH 2.0 to 10.0 (Fig. 2b).

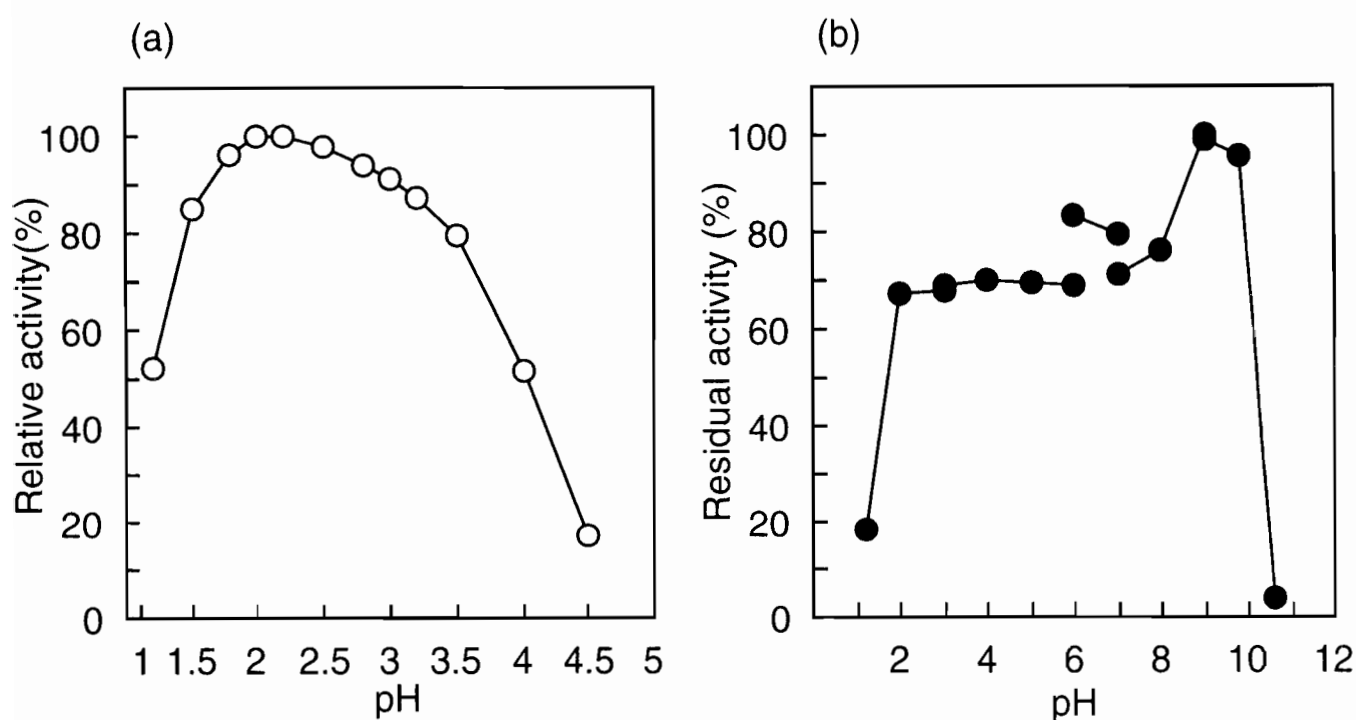


Fig. 2. Effects of pH on the Activity (a) and the Stability (b) of ACPase II.

(a) The enzyme activities were assayed at pH 1.2-4.5 (0.2 M glycine-HCl buffer).

(b) The enzyme was incubated at various pHs at 37°C for 60 min, and the residual activities were assayed at pH 2.0. The enzyme activity was measured using G6P as a substrate. The highest activity was designated as 100%. Buffers; 0.2 M of glycine-HCl pH 1.2-3.0), sodium acetate (pH 3.0-6.0), MES-NaOH (pH 6.0-7.0), Tris-HCl (pH 7.0-9.0), and glycine-NaOH (pH 9.0-10.6).

1.3.5 Effects of temperature on activity and stability of ACPase II

The effects of temperature on activity and stability of ACPase II are shown in Fig. 3. The enzyme activity was measured using G6P as a substrate, because G6P was more stable than pNPP at high temperatures, above 40°C. The optimum temperature for the activity was 60°C (Fig. 3a), and the enzyme was stable up to 50°C (Fig. 3b).

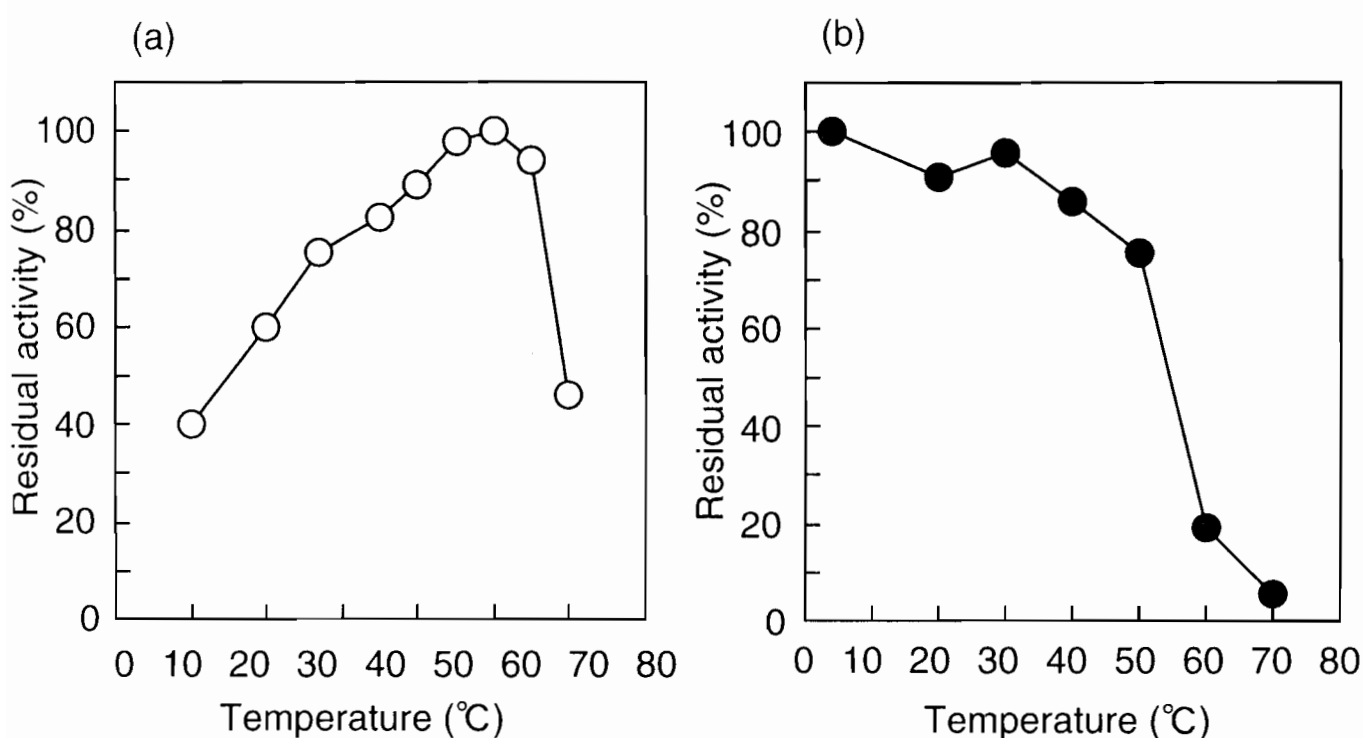


Fig. 3. Effects of Temperature on the Activity (a) and the Stability (b) of ACPase II.

(a) The enzyme activities were assayed at various temperatures (20-70°C) at pH 2.0. (b) The enzyme was incubated at various temperatures (4-70°C) for 30 min, and the residual activities were assayed at 37°C at pH 2.0. The enzyme activity was measured using G6P as a substrate. The highest activity was designated as 100%.

1.3.6 Substrate specificity

The substrate specificity of ACPase II was examined by using a number of phosphate esters (Table 2). The enzyme was highly active toward β -naphthyl phosphate, fructose-1,6-bisphosphate, ADP, ATP, phosphoenolpyruvate, pyrophosphate, and polyphosphate, but scarcely active toward bis(*p*-nitrophenyl)phosphate, fructose 6-phosphate, and phytic acid.

Table 2. Substrate Specificity of ACPase II

Substrate	Relative activity (%)
pNPP	100.0
β -Naphthyl phosphate	111.0
α -Glycero phosphate	48.1
β -Glycero phosphate	60.5
Bis(<i>p</i> -nitrophenyl) phosphate	2.4
Glucose 1-phosphate	53.5
Glucose 6-phosphate	64.5
Fructose 6-phosphate	16.2
Fructose 1,6-bisphosphate	103.7
AMP	25.1
ADP	83.1
ATP	91.1
Phosphoenol pyruvate	82.8
Pyrophosphate	163.7
Polyphosphate*	103.3
Phytic acid**	8.7

ACPase II was incubated with each substrate at 37°C for 15 min. The enzyme activity was assayed by measuring the amount of released inorganic phosphate, and the relative activity was expressed as a percentage of the activity on pNPP.

*substrate concentrations were 0.7%. **substrate concentrations were 1.5%.

1.3.7 Effects of various metal ions and chemicals

The effects of various metal ions and chemical reagents on the activity of the ACPase II are shown in Table 3. The enzyme activity was strongly inhibited by Fe^{3+} and F^- . L(+)-tartrate which is a competitive inhibitor of several acid phosphatases from animal sources (Kilsheimer and Axlerod, 1956, McTigue and Van Etten, 1978) did not inhibit the enzyme.

1.3.8 Action of ACPase II on PO-1

The action pattern of ACPase II on PO-1 was investigated. The HPAEC chromatograms of the standard PO-1 and the PO-1 after the enzyme reaction were shown in Fig. 4. As shown in Fig. 4b, PO-1 was a mixture of 3³-phosphoryl maltotetraose (peak J) and 3⁴-phosphoryl maltopentaose (peak K) for the 3-phosphoryl oligosaccharides, and 6³-phosphoryl maltotriose (peak L), 6²-phosphoryl maltotriose (peak L), 6³-phosphoryl maltotetraose (peak M), and 6⁴-phosphoryl maltopentaose (peak N) for the 6-phosphoryl oligosaccharides. After the ACPase II treatment, 6-phosphoryl oligosaccharides almost disappeared, and maltotriose, maltotetraose, and maltopentaose were produced from 6³-phosphoryl maltotriose and 6²-phosphoryl maltotriose, 6³-phosphoryl maltotetraose, and 6⁴-phosphoryl maltopentaose, respectively (Fig. 4c). Three major peaks at the retention time of 10-15 min in Fig. 4c were certainly confirmed as neutral sugars (maltotriose, maltotetraose, and maltopentaose). On the other hand, 3-phosphoryl oligosaccharides were barely dephosphorylated.

Figure 5 shows the course of the reaction of ACPase II on 3-phosphoryl oligosaccharides and 6-phosphoryl oligosaccharides that existed in PO-1. As the reaction continued, 6-phosphoryl oligosaccharides gradually decreased and 6³-phosphoryl maltotetraose and 6⁴-phosphoryl maltopentaose decreased at the same rate. After 6 h, 6³-phosphoryl maltotetraose and 6⁴-phosphoryl maltopentaose decreased to less than 5% and the mixture of 6³-phosphoryl maltotriose plus

Table 3. Effects of various Metal Ions and Chemical Reagents on the Activity of ACPase II

Metal ions and reagents	Conc. (mM)	Relative activity (%)
None	-	100.0
Al ³⁺	10	80.8
Ca ²⁺	10	100.7
Co ²⁺	10	100.5
Cu ²⁺	10	96.6
EDTA-2Na	10	111.5
Fe ²⁺	10	27.4
	1	78.3
Fe ³⁺	10	0.0
	1	0.0
K ⁺	10	100.9
Li ²⁺	10	97.9
Mg ²⁺	10	98.5
Mn ²⁺	10	98.8
Na ⁺	10	89.9
Ni ²⁺	10	101.4
Zn ²⁺	10	101.3
Boric acid	10	95.0
D(-)-tartrate	10	98.5
L(+)-tartrate	10	93.7
DL-tartrate	10	94.3
KF	10	17.7
NaF	10	0.0
Molybdate	10	0.0
<i>p</i> -Chloromercuribenzone	0.1	96.6
SDS	10	0.0
<i>N</i> -Acethylimidazol	1	113.3
<i>N</i> -Bromosuccinimide	1	92.9
2-Hydroxy-5-nitrobenzylbromide	1	0.0
Dithiothreitol	1	108.6
Iodoacetic acid	1	104.3

ACPase II was incubated at 37°C for 15 min in the presence of various metal ions and chemical reagents (final concentration described above). The residual activity was assayed at 37°C using G6P as a substrate, and the relative activity was expressed as a percentage of the enzyme activity in the absence of metal ions and chemical reagents.

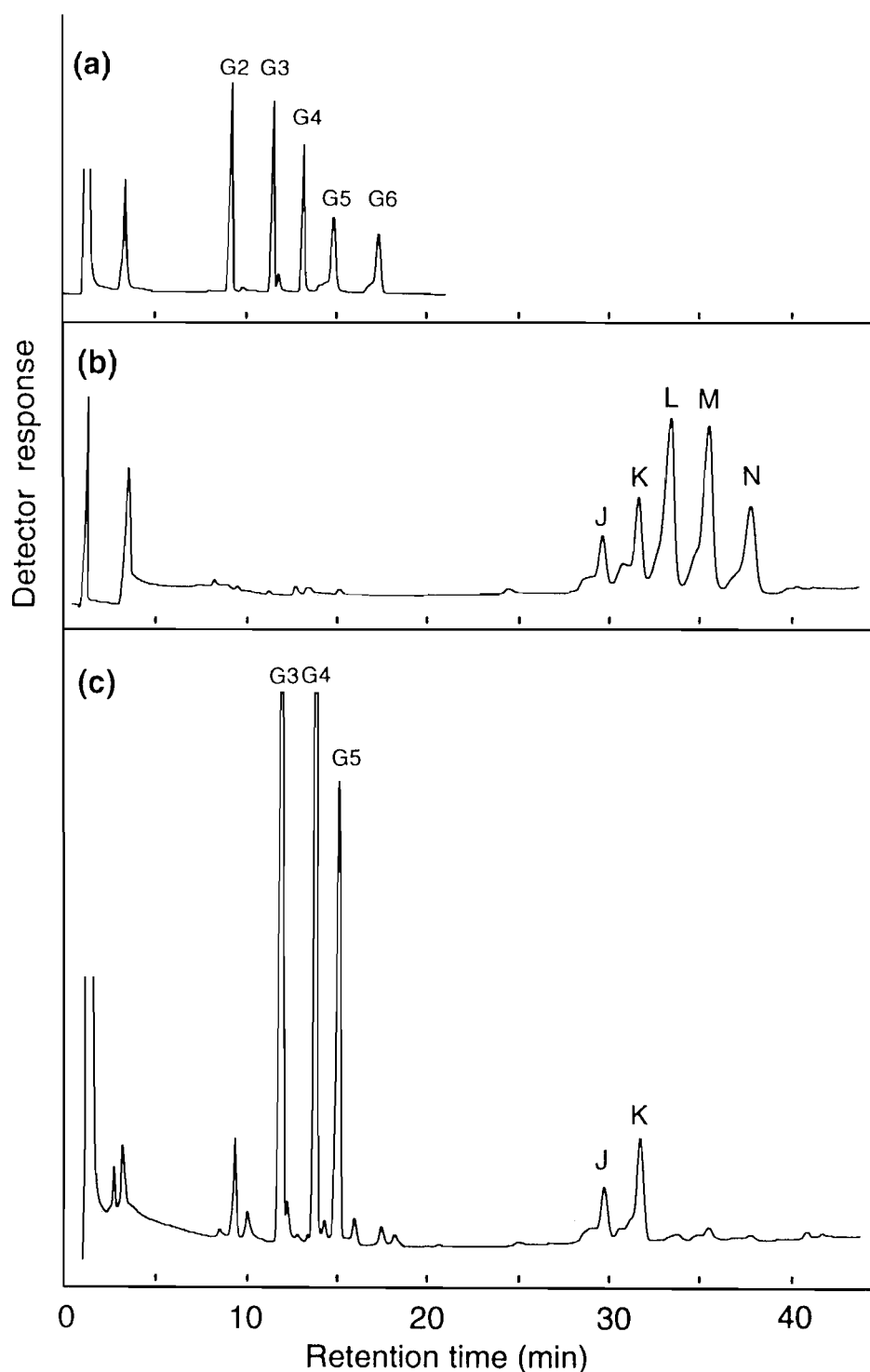


Fig. 4. HPAEC Chromatograms of Reaction of ACPase II on PO-1.

(a) standard maltooligosaccharides, (b) PO-1 fraction, (c) PO-1 fraction after reaction of ACPase II. Peak J, 3³-phosphoryl maltotetraose; K, 3⁴-phosphoryl maltopentaose; L, 6³-phosphoryl maltotriose and 6²-phosphoryl maltotriose; M, 6³-phosphoryl maltotetraose; N, 6⁴-phosphoryl maltopentaose. G2, maltose; G3, maltotriose, G4, maltotetraose; G5, maltopentaose; G6, maltohexaose.

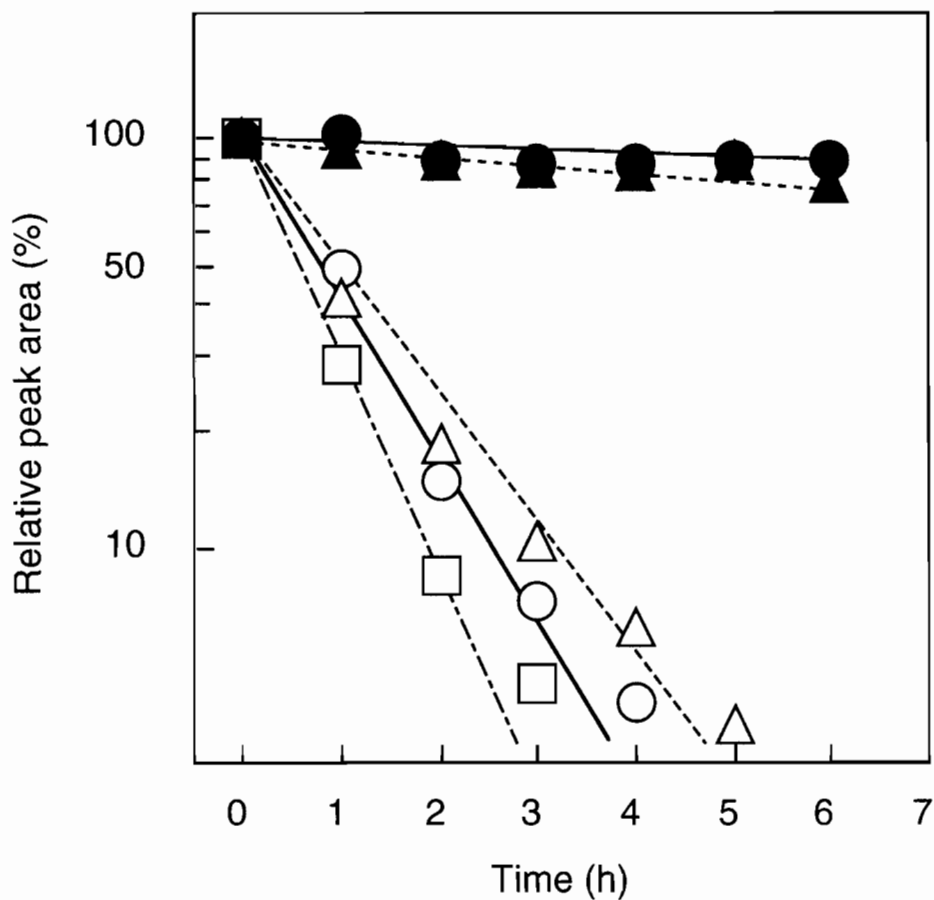


Fig. 5. Course of Reaction of ACPase II on 3-Phosphoryl Oligosaccharides and 6-Phosphoryl Oligosaccharides.

ACPase II was incubated with PO-1 fraction at 37°C. At intervals, the remaining phosphoryl oligosaccharides in the reaction mixtures was analyzed with HPAEC. The relative peak area is indicated as a percentage against the initial amount of the substrate. Vertical line is expressed on a logarithmic scale. Symbols: ▲, 3³-phosphoryl maltotetraose; ●, 3⁴-phosphoryl maltopentaose; □, 6³-phosphoryl maltotriose and 6²-phosphoryl maltotriose; △, 6³-phosphoryl maltotetraose; ○, 6⁴-phosphoryl maltopentaose.

6²-phosphoryl maltotriose (Fig. 4b; peak L) disappeared within 4 h. On the contrary, both 3³-phosphoryl maltotetraose and 3⁴-phosphoryl maltopentaose were barely dephosphorylated, and the rate of the decrease of 3³-phosphoryl maltotetraose was a little higher than 3⁴-phosphoryl maltopentaose. Even after 6 h of reaction, about 80% of 3³-phosphoryl maltotetraose and 90% of 3⁴-phosphoryl maltopentaose still remained in the mixture.

1.3.9 Properties of ACPase I

A. niger KU-8 produced another type of intracellular acid phosphatase (ACPase I). Some properties of ACPase I were preliminarily investigated using partially purified enzyme. ACPase I poorly hydrolyzed both 3-phosphoryl oligosaccharides and 6-phosphoryl oligosaccharides, although it was a major component of intracellular acid phosphatases in *A. niger* KU-8 and had higher activity for pNPP than did ACPase II. The molecular weight of the enzyme was estimated to be about 77 kDa by SDS-PAGE (data not shown). As shown in Fig. 6, the optimum pH and pH stability for the enzyme activity of ACPase I was 3.0 and 3.0-8.0, respectively, and optimum temperature was 60°C and ACPase I was stable up to 60°C. The enzyme activity was inhibited 84% by Al³⁺, and 38% by Fe³⁺ (at 1 mM) and 65% by Fe²⁺ (at 1 mM), and completely by Fe³⁺ (at 10 mM), KF, NaF, molybdate, SDS, and 2-hydroxy-5-nitrobenzylbromide. The enzyme was also insensitive to sulfhydryl reagents and EDTA, but sensitive to L(+)-tartrate (56% Inhibition). As to substrate specificity, ACPase I had low activities toward G6P (32% of that for pNPP), fructose 1,6-bisphosphate (25%), and polyphosphate (35%), although ACPase II hydrolyzed them up to 65%, 104%, and 103%, respectively. In addition, ACPase I hydrolyzed AMP (76%) followed by ADP (65%) rather than ATP (25%), which was contrary to ACPase II, and showed no activity toward phytic acid.

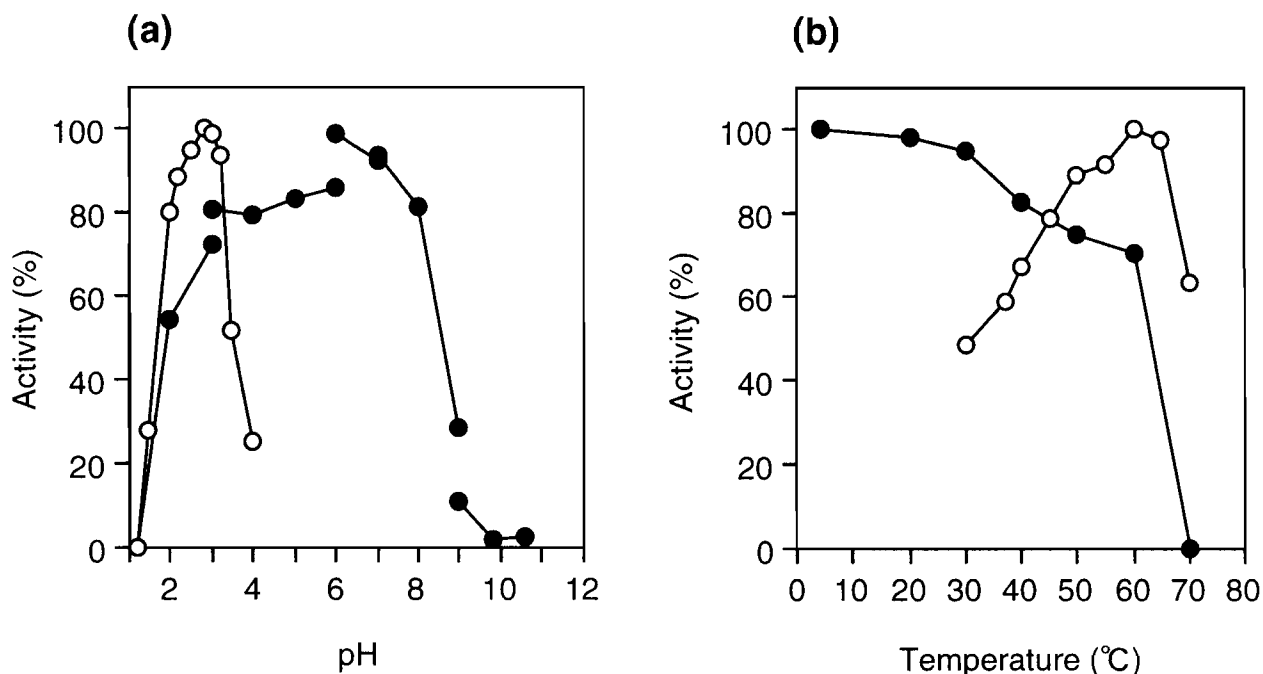


Fig. 6. Effects of pH (a) and Temperature (b) on the Activity (○) and Stability (●) of ACPase I.

(a) Relative activity of the enzyme were assayed at pH 1.2-4.5 (0.2 M glycine-HCl buffer) using G6P as a substrate. To examine the pH stability, the enzyme was incubated at various pHs at 37°C for 60 min, and the residual activities were assayed at pH 3.0. The highest activity was designated as 100%. Buffers; 0.2 M glycine-HCl (pH 1.2-3.0), sodium acetate (pH 3.0-6.0), MES-NaOH (pH 6.0-7.0), and Tris-HCl (pH 7.0-9.0), and glycine-NaOH (pH 9.0-10.6).

(b) Relative activity of the enzyme were assayed at various temperatures (30-70 °C) at pH 3.0 using G6P as a substrate. To examine the thermal stability, the enzyme was incubated at various temperatures (4-70°C) for 30 min, and the residual activity was assayed at 37°C at pH 3.0. The highest activity was designated as 100%.

1.4 Discussion

In this section, the author tried to separate enzymatically 3-phosphoryl oligosaccharides and 6-phosphoryl oligosaccharides for the better understanding of their properties. The author tested several phosphatases that were commercially available, but there had been no phosphatases which had distinct substrate specificities toward C-3 or C-6 phosphate groups of PO-1. Therefore, the author screened for the microorganisms which produced a phosphatase specific for 6-phosphoryl oligosaccharides of PO-1 and succeeded in isolating of *A. niger* KU-8, which produced an acid phosphatase of interest.

A. niger KU-8 produced two types of acid phosphatase (ACPase I and II). ACPase I had a weak reaction and no specificity for 3-phosphoryl oligosaccharides and 6-phosphoryl oligosaccharides, although it was a major component of intracellular acid phosphatases of KU-8 and had higher activity for pNPP. On the other hand, ACPase II preferentially dephosphorylated the C-6 phosphate groups and scarcely the C-3 phosphate groups of PO-1, although it was a minor component and has a less activity for pNPP relative to ACPase I.

From the estimation of molecular weight, it is suggested that the native ACPase II has a tetramer structure. Other acid phosphatases having similar molecular weight already reported. For example, Shimada *et al.* (1974, 1977) reported a cell wall-bound acid phosphatase from *A. niger* U20-2-5 with a molecular weight of 260 kDa, and Zyla (1990) also described an intracellular acid phosphatase from *A. niger* 'Z' (230 kDa), though both of the numbers of subunits had not been clarified. An intracellular acid phosphatase from *A. niger* U20-2-5 (Shimada *et al.*, 1974, 1977) had a molecular weight of 310 kDa (native) and 89 kDa (subunit). In contrast, a monomeric structure of the native enzyme was reported for *Aspergillus ficuum* (Ullah and Cummins, 1988) and *Penicillium*

chrysogenum (Haas *et al.*, 1991). Furthermore, acid phosphatases from *Saccharomyces cerevisiae* (Barbaric *et al.*, 1984) and *A. ficuum* (Ullah and Cummins, 1987) are known to be dimeric.

The pH activity profile of ACPase II suggested that the enzyme was active even at extremely low pH and had good pH stability over a wide pH range.

ACPase II was not dependent on free sulfhydryl groups, since sulfhydryl reagents of *p*-chloromercuribenzoate, dithiothreitol, and iodoacetic acid had no effect on the enzyme activity. ACPase II was also insensitive to EDTA, indicating metal ion(s) do not participate in the enzyme activity. Sodium fluoride is known to be a typical inhibitor of acid phosphatases from bacteria (Hollander, 1971) and the activity of ACPase II was also inhibited completely.

From the results of the action on PO-1, the most remarkable substrate specificity of ACPase II described here is that the enzyme preferentially dephosphorylates the C-6 phosphate groups of PO-1. It suggests that ACPase II can distinguish between the binding positions of these phosphate groups. Comparing the dephosphorylation rate of 3-phosphoryl oligosaccharides with 6-phosphoryl oligosaccharides having the same DP, it seems reasonable to suppose that the difference of the ACPase II reactivity with them is due to the difference of binding position of phosphate groups of PO-1. Furthermore, this result indicates a possibility that the dephosphorylation rate of 6-phosphoryl oligosaccharides by ACPase II is dependent on the DP of them, namely, the lower DP of 6-phosphoryl oligosaccharides, the faster ACPase II dephosphorylates phosphate groups of 6-phosphoryl oligosaccharides. A similar tendency was also observed in the case of dephosphorylation of 3-phosphoryl oligosaccharides, although the difference of the dephosphorylation rates between them was small.

In spite of the strict specificity toward the phosphate groups in the

PO-1 fraction, ACPase II showed broad specificity for other phosphate esters. ACPase II hydrolyzed ATP, pyrophosphate, and polyphosphate as well as phosphate monoesters like pNPP. In addition, ACPase II scarcely hydrolyzed phytic acid. This result indicates that a possibility that ACPase II is a phytase would be ruled out.

1.5 Summary

Aspergillus niger strain KU-8 produced two types of intracellular acid phosphatase (EC 3.1.3.2, ACPase); ACPase I and II. ACPase II preferentially dephosphorylated 6-phosphoryl oligosaccharides rather than 3-phosphoryl oligosaccharides. The molecular weight of the enzyme was estimated as 66 kDa by SDS-PAGE and about 260 kDa by gel filtration, implying the active form to be a tetramer. The optimum pH and temperature of the enzyme were 2.0 and 60°C, respectively. ACPase II was stable below 50°C for 30 min and at pH 2.0-10.0 for 60 min. In spite of the strict specificity toward 6-phosphoryl oligosaccharides in the PO-1 fraction, ACPase II was able to hydrolyze fructose 1,6-bisphosphate, ATP, pyrophosphate, and polyphosphate as well as pNPP and G6P, a broad substrate specificity.

SECTION 2

Substrate selectivity of acid phosphatase II in *Aspergillus niger* KU-8 on C-3 and C-6 phosphate groups of phosphoryl oligosaccharides

2.1 Introduction

Phosphoryl oligosaccharides (POs) are potato starch hydrolysates, and have phosphate groups which binding ratio of C-3 to C-6 is approximately 3 to 7. In the previous section (Chapter I, section 1) the author described the characteristics of a novel intracellular acid phosphatase, ACPase II, from *Aspergillus niger* KU-8. This enzyme preferentially dephosphorylated phosphate groups of 6-phosphoryl oligosaccharides rather than those of 3-phosphoryl oligosaccharides.

Little is known of the properties of C-3 phosphate group because of the difficulty in preparation of standard samples of saccharides phosphorylated at C-3. Glucose 3-phosphate has been detected by acid hydrolysis of potato starch (Tabata and Hizukuri, 1971, Blennow *et al.*, 1998). However, because of the inherent instability of the phosphoester linkage at C-3 compared to the C-6 position, acid hydrolysis results in the fatal loss of saccharides phosphorylated at C-3. Therefore, the preparation of C-3 phosphoryl saccharide including glucose 3-phosphate by acid hydrolysis of potato starch is essentially impossible.

In this section, 3²-phosphoryl maltotriose and 6²-phosphoryl maltotriose were prepared from PO-1 fraction as each standard sample of 3-phosphoryl oligosaccharides and 6-phosphoryl oligosaccharides with the combination of fractionation with ODS column chromatography and treatment with α -amylase (bacterial saccharifying α -amylase (BSA, EC 3.2.1.1) or neopullulanase (NPL, EC 3.2.1.135)). BSA hydrolyzes α -1,4-glucosidic linkages present at non-reducing end site and produces

glucose, maltose, maltotriose and various branched oligosaccharides from starch (Yamamoto, 1988). NPL hydrolyzes both α -1,4 and α -1,6 linkages and produces panose (6²-*O*- α -glucosyl-maltose), glucose and maltose from pullulan (Kuriki *et al.*, 1988, 1996, Kuriki and Okada, 1995).

Then, the kinetic parameters of ACPase II for 3²-phosphoryl maltotriose and 6²-phosphoryl maltotriose were compared. In addition, the kinetic parameters of ACPase I for them also measured. These two oligosaccharides have the same maltotriose moiety and differ only in the binding position of the phosphate group.

2.2 Materials and Methods

2.2.1 Enzymes

Bacterial saccharifying α -amylase (BSA) from *Bacillus subtilis* was purchased from Nagase Biochemicals Co. (Fukuchiyama, Japan). Neopullulanase (NPL) from *Bacillus stearothermophilus* (Kuriki *et al.*, 1988) was purified in the author's laboratory. ACPase II was purified from the cell-free extract of *A. niger* KU-8 as described in previous section. ACPase I was used in the partially purified form.

2.2.2 Other materials

p-Nitrophenyl phosphate (pNPP) and fructose 6-phosphate and were purchased from Wako Pure Chemical Industries. Glucose 6-phosphate (G6P) was purchased from Nacalai Tesque. Glucose 1-phosphate (G1P) was purchased from Boehringer Mannheim GmbH. Daisopak SP-120-10-ODS-B column was purchased from Daiso Co. (Osaka, Japan). All other chemicals and materials used were of analytical or commercial grade. PO-1 was prepared from potato starch hydrolysate.

2.2.3 Preparation of 3²-phosphoryl maltotriose and 6²-phosphoryl maltotriose

2.2.3.1 Fractionation of 3-phosphoryl oligosaccharides and 6-phosphoryl oligosaccharides by Daisopak ODS-B column chromatography

Fractionation of 3-phosphoryl oligosaccharides and 6-phosphoryl oligosaccharides from PO-1 were done by HPLC with an ODS preparative column under following conditions: column, Daisopak SP-120-10-ODS-B (3 ϕ x 50 cm); mobile phase, 10 mM K₂HPO₄-H₃PO₄ buffer (pH 3.5); detection, reflective index detector; temperature, ambient; flow rate, 20 mL/min; injection volume, 1.5 mL (200 mg/mL PO-1 solution). The total amount of PO-1 injected was 18.0 g. The 3- and 6-phosphoryl oligosaccharides eluted from the column were fractionated by hand. Fractionation of them was confirmed by high performance anion-exchange chromatography (HPAEC) as described in Chapter I, section 1.

2.2.3.2 Preparation of 3²-phosphoryl maltotriose

The 3-phosphoryl oligosaccharides fraction were collected and concentrated with a rotary evaporator. The phosphate buffer used for ODS chromatography was removed with activated charcoal chromatography, and 3-phosphoryl oligosaccharides were eluted by 50% ethanol. Ethanol was removed with a rotary evaporator, and the mixture of 3-phosphoryl oligosaccharides (estimated at about 4.5 g) was treated with 1000 units of BSA in 0.2 M acetate buffer (pH 5.5) at 37°C for 16 h, and 3²-phosphoryl maltotriose was prepared. Neutral saccharides produced by BSA reaction were removed with the second activated charcoal chromatography. After the reaction, BSA was removed by ultrafiltration.

2.2.3.3 Preparation of 6²-phosphoryl maltotriose

The preparation step of 6²-phosphoryl maltotriose was almost the same

as 3²-phosphoryl maltotriose. After the first activated charcoal chromatography for the removal of phosphate buffer used at ODS chromatography, the mixture of 6-phosphoryl oligosaccharides was treated with 1000 units of NPL in 0.2 M acetate buffer (pH 6.0) at 37°C for 16 h. Since NPL produced 6²-phosphoryl maltotriose and a small amount of 6²-phosphoryl maltose, ODS chromatography was used again for the removal of 6²-phosphoryl maltose. Neutral saccharides produced by the NPL reaction were removed with a second activated charcoal chromatography. After the reaction, NPL was removed by ultrafiltration with Ultrafree-MC (nominal molecular weight limit of 30,000, Millipore).

2.2.4 Assay of bacterial saccharifying α -amylase (BSA) activity

The assay of BSA activity was according to the 3,5-dinitrosalicylic acid method (Kuriki *et al.*, 1996). The reaction mixture (200 μ L) consisted of 0.5% soluble starch (MERCK, Darmstadt, Germany) in 20 mM of sodium acetate buffer (pH 5.5) and the enzyme. The reaction was stopped after 10 min of incubation at 55 °C by the addition of 200 μ L of 3,5-dinitrosalicylic acid reagent. The reagent was prepared by mixing 0.4 M NaOH, 22 mM 3,5-dinitrosalicylic acid and 1.1 M potassium sodium (+)-tartrate tetrahydrate. One unit of enzyme activity was defined as the amount of enzyme that released 1 μ mol of reducing sugar as glucose per min under assay condition.

2.2.5 Assay of neopullulanase (NPL) activity

The assay procedure of NPL activity was followed by the same method as described in assay of BSA activity, except for the use of 1% pullulan as a substrate (Kuriki *et al.*, 1996).

2.2.6 Assay of acid phosphatase (ACPase) activity

The assay of ACPase II activity was followed by the method as

described in Chapter I, section 1.

2.2.7 Measurement of Michaelis constant (K_m) and molecular activity (k_{cat})

The K_m and k_{cat} of ACPase II for pNPP, G1P, G6P, PO-1, 3²-phosphoryl maltotriose and 6²-phosphoryl maltotriose were measured. One unit of enzyme activity was defined as 1 μ mol of inorganic phosphate release from these substrates per min. In addition, the K_m of ACPase I in partial purified form for pNPP, G6P and G1P were also measured.

2.3 Results

2.3.1 Fractionation of 3-phosphoryl oligosaccharides and 6-phosphoryl oligosaccharides

Figure 1 shows the chromatogram of POs fractionated by Daisopak ODS-B column and that of each phosphoryl oligosaccharides by HPAEC. POs were eluted from the ODS-B column in order of 6-phosphoryl oligosaccharides and 3-phosphoryl oligosaccharides, opposite to HPAEC.

2.3.2 Preparation of 3²-phosphoryl maltotriose

3-Phosphoryl oligosaccharides fractionated by Daisopak ODS-B column were treated with bacterial saccharifying α -amylase (BSA) after removal of phosphate buffer used in the ODS chromatography. BSA could hydrolyze 3⁴-phosphoryl maltotetraose and 3⁴-phosphoryl maltopentaose, and then 1 mol of glucose and maltose were released from the reducing end, respectively. Figure 2 shows the chromatogram of result of BSA reaction on 3-phosphoryl oligosaccharides by HPAEC. Neutral saccharides produced by BSA reaction were removed with active charcoal chromatography. Finally, 0.45 g of 3²-phosphoryl maltotriose was obtained because of sample loss during preparation.

2.3.3 Preparation of 6²-phosphoryl maltotriose

6-Phosphoryl oligosaccharides fractionated by Daisopak ODS-B column were treated with neopullulanase (NPL) after removal of phosphate buffer used in the ODS chromatography. NPL could hydrolyze 6³-phosphoryl maltotriose, 6⁴-phosphoryl maltotetraose and 6⁴-phosphoryl maltopentaose, and then 1 mol of glucose, glucose and maltose were released from the reducing end, respectively. Figure 3 shows the chromatogram of result of NPL reaction on 6-phosphoryl oligosaccharides by HPAEC. Neutral saccharides produced by NPL reaction were

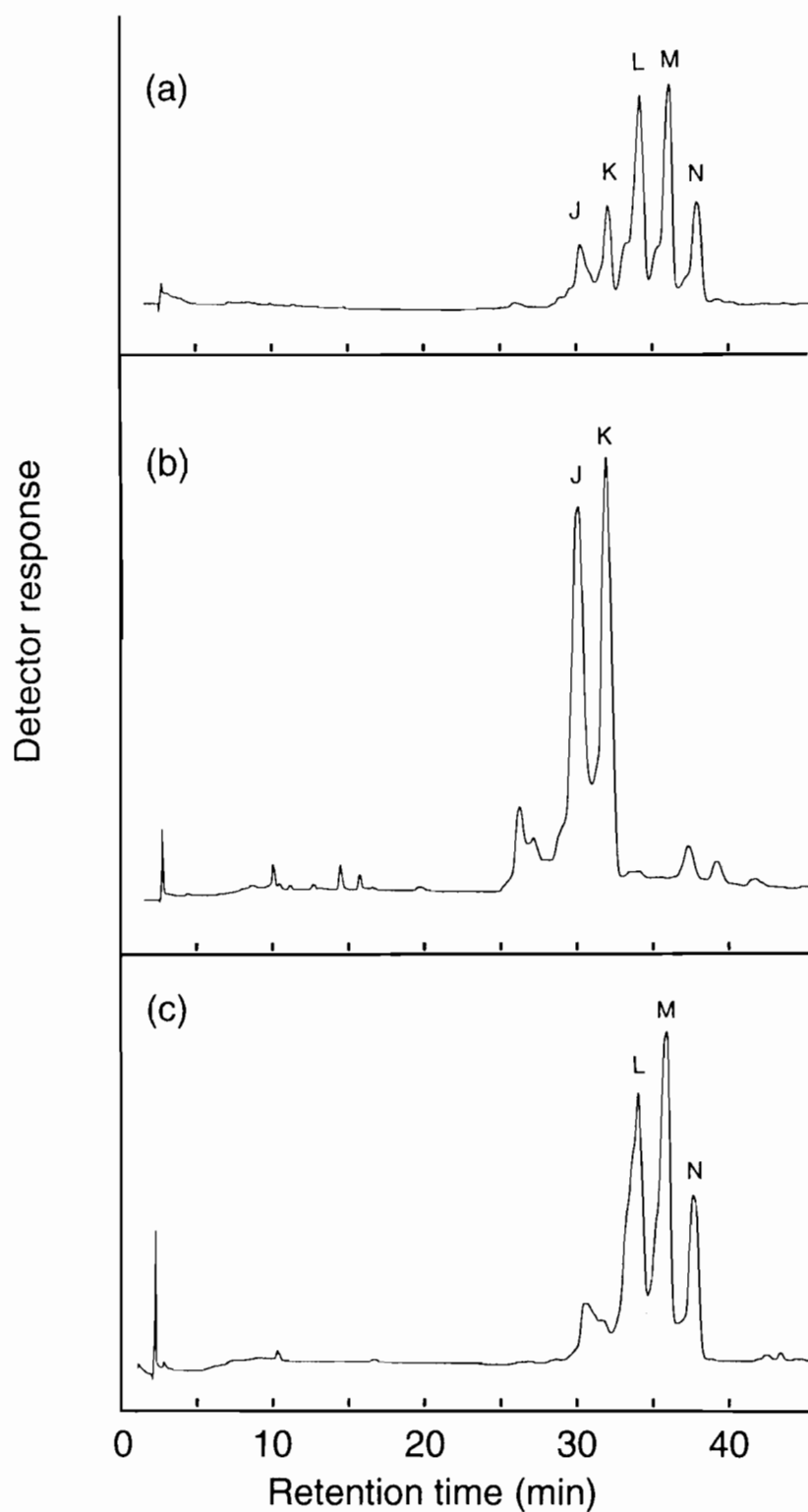


Fig. 1. HPAEC Chromatograms of 3-Phosphoryl Oligosaccharides and 6-Phosphoryl Oligosaccharides after Fractionation by Daisopak ODS-B Column.

(a) PO-1 fraction, (b) 3-phosphoryl oligosaccharides, (c) 6-phosphoryl oligosaccharides. Peak J, 3³-phosphoryl maltotetraose; K, 3⁴-phosphoryl maltopentaose; L, 6³-phosphoryl maltotriose and 6²-phosphoryl maltotriose; M, 6³-phosphoryl maltotetraose; N, 6⁴-phosphoryl maltopentaose.

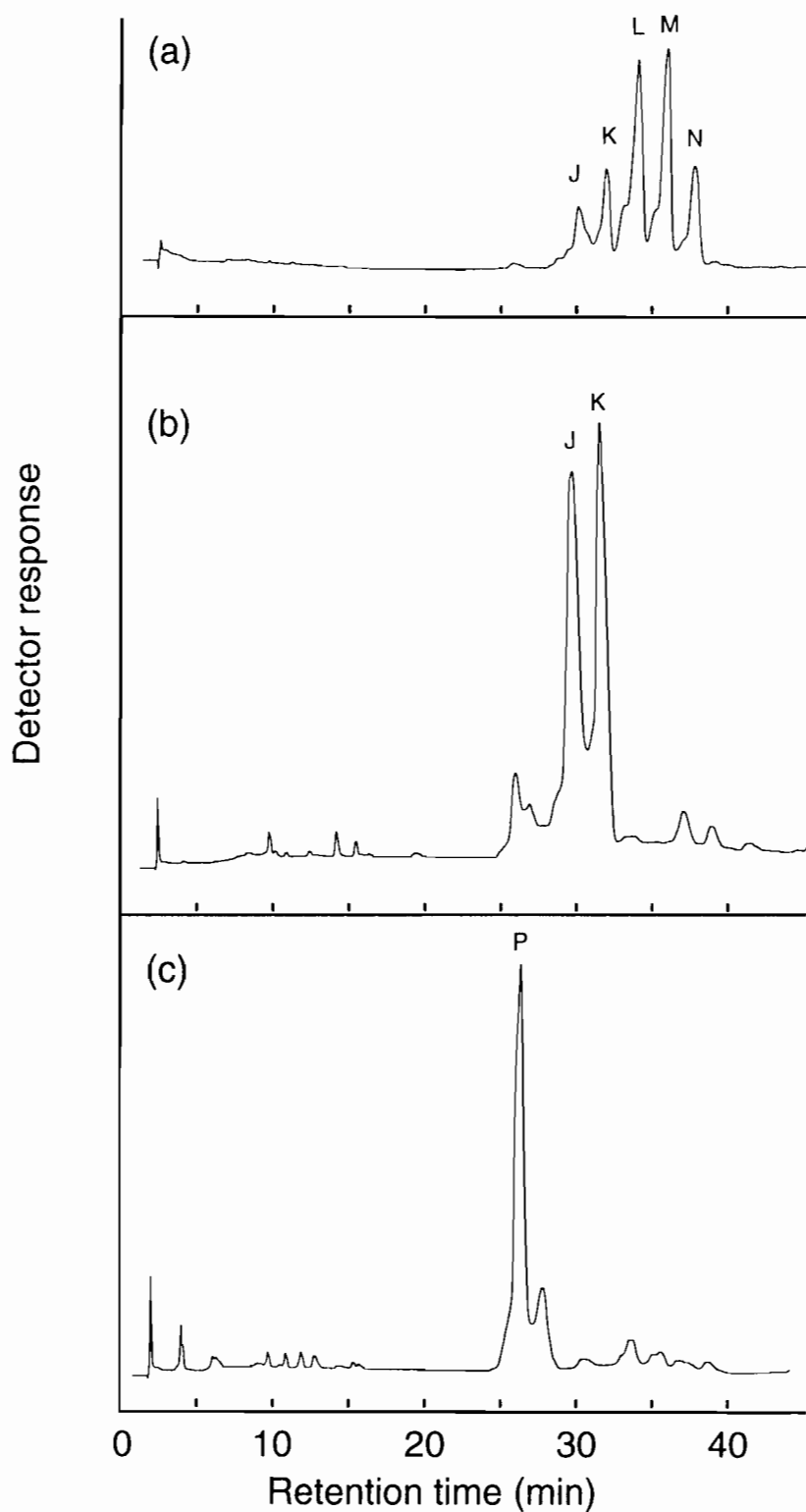


Fig. 2. HPAEC Chromatograms of 3-Phosphoryl Oligosaccharides after BSA Treatment.

(a) PO-1 fraction, (b) 3-phosphoryl oligosaccharides, (c) 3-phosphoryl oligosaccharides after BSA treatment. Peak J, 3³-phosphoryl maltotetraose; K, 3⁴-phosphoryl maltopentaose; L, 6³-phosphoryl maltotriose and 6²-phosphoryl maltotriose; M, 6³-phosphoryl maltotetraose; N, 6⁴-phosphoryl maltopentaose; P, 3²-phosphoryl maltotriose. BSA, bacterial saccharifying α -amylase.

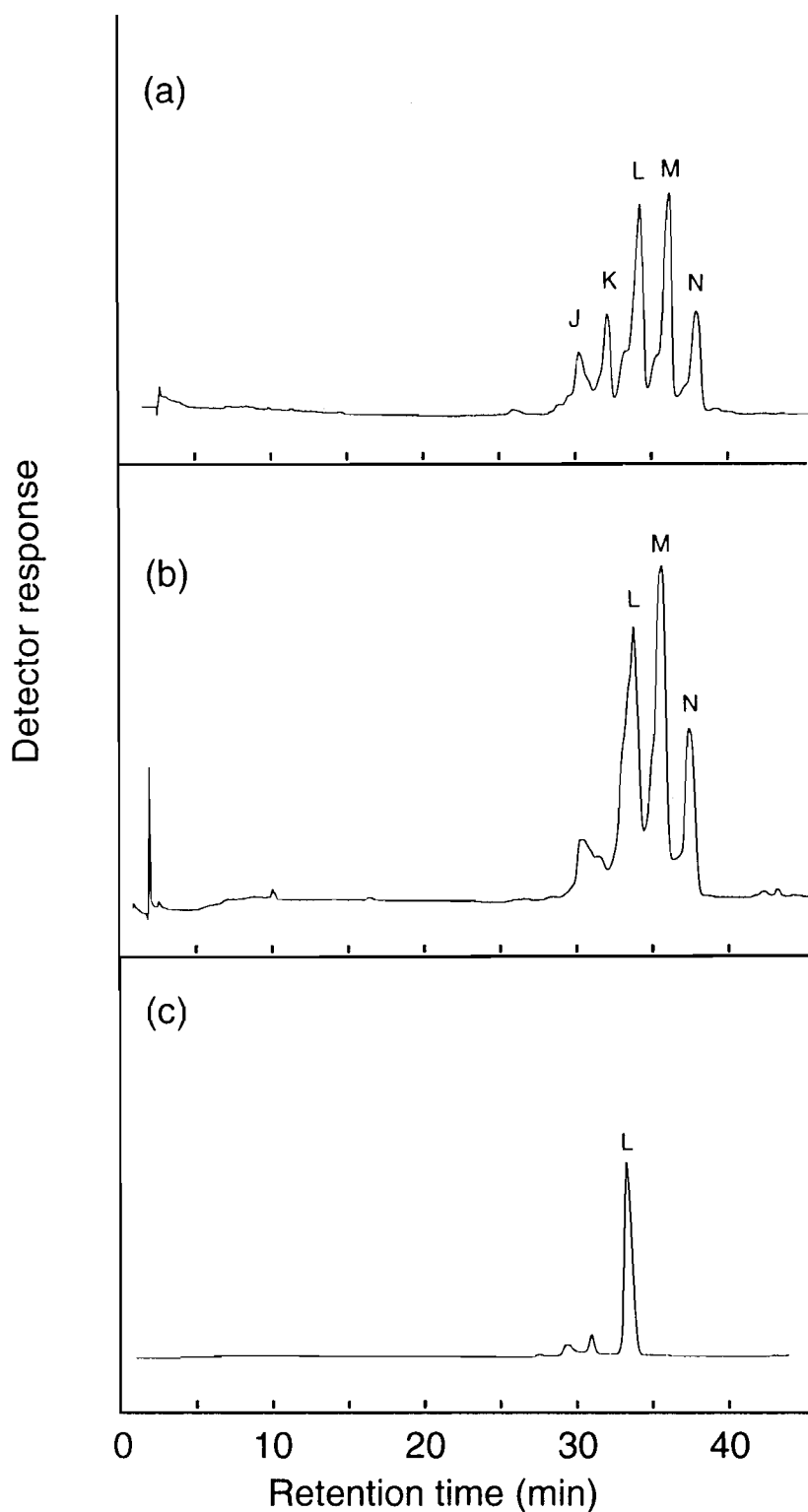


Fig. 3. HPAEC Chromatograms of 6-Phosphoryl Oligosaccharides after NPL Treatment.

(a) PO-1 fraction, (b) 6-phosphoryl oligosaccharides, (c) 6-phosphoryl oligosaccharides after NPL treatment. Peak J, 3³-phosphoryl maltotetraose; K, 3⁴-phosphoryl maltopentaose; L, 6³-phosphoryl maltotriose and 6²-phosphoryl maltotriose; M, 6³-phosphoryl maltotetraose; N, 6⁴-phosphoryl maltopentaose. NPL, neopullulanase.

removed with active charcoal chromatography. Finally, 2.70 g of 6²-phosphoryl maltotriose was obtained.

The hydrolytic reactions of BSA and NPL on maltooligosaccharides and PO-1 fraction are illustrated in Table 1 and Fig. 4, respectively.

Table 1. Cleavage Points of BSA and NPL on Maltooligosaccharides

			
BSA	↑	↑ ↑	↑ ↑
NPL	↑	↑	↑ ↑

Long and short arrows indicate the extent of the enzyme reactions, fast and slow, respectively. ○, glucosyl residue; ◐, reducing glucosyl residue.

BSA, bacterial saccharifying α -amylase; NPL, neopullulanase.

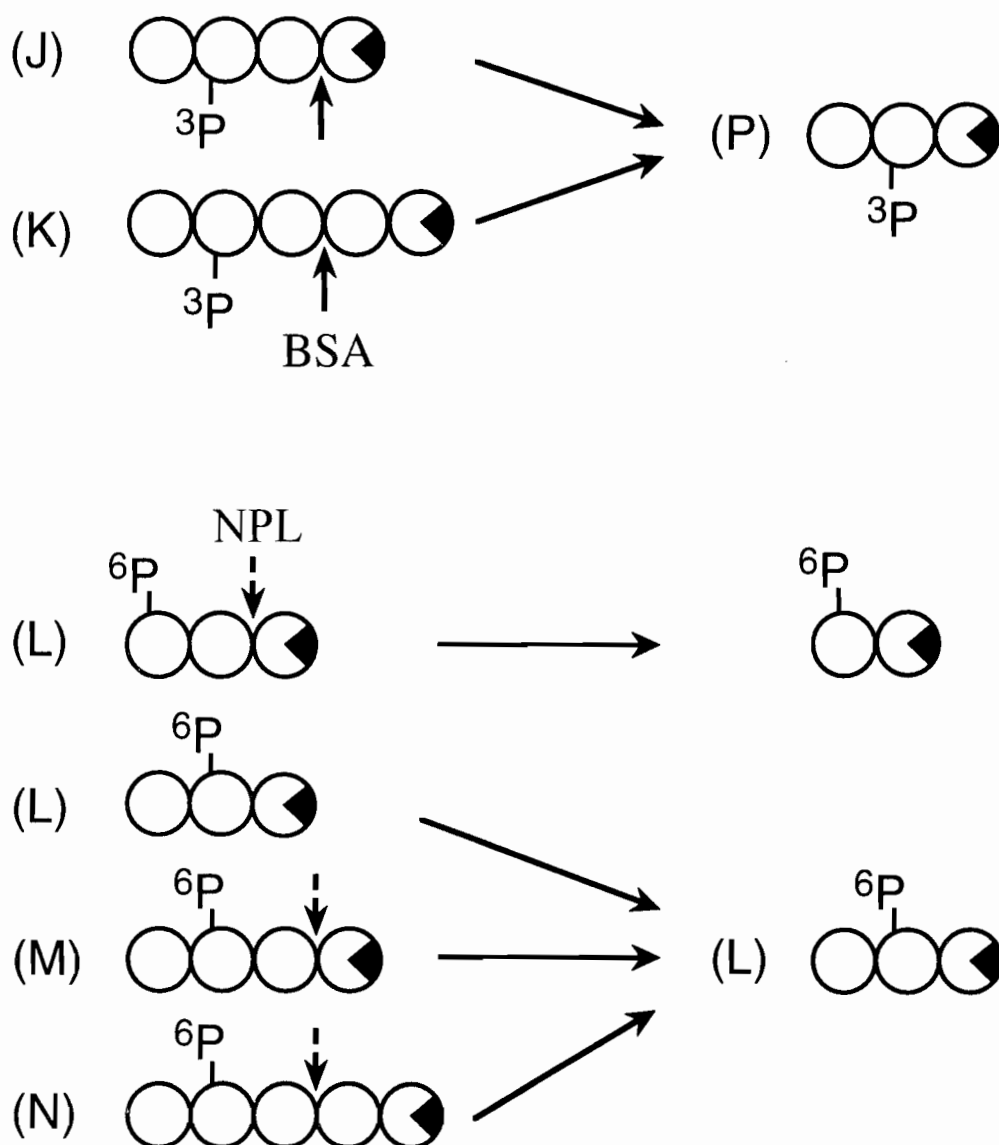


Fig. 4. Cleavage points of 3-Phosphoryl Oligosaccharides by BSA and 6-Phosphoryl Oligosaccharides by NPL.

Symbols: 3P and 6P , phosphate esters bound at C-3 and C-6 of glucosyl residues, respectively. $\bigcirc$, glucosyl residue; $\bigcirc$ (half-black), reducing glucosyl residue. J-N and P correspond to the peaks in Fig. 1-3.

BSA, bacterial saccharifying α -amylase; NPL, neopullulanase.

2.3.4 Measurement of Michaelis constant (K_m) and molecular activity (k_{cat})

The Michaelis constant (K_m) and molecular activity (k_{cat}) of ACPase II for pNPP, G1P, G6P, PO-1, 3²-phosphoryl maltotriose, and 6²-phosphoryl maltotriose were obtained from Lineweaver-Burk plots (Fig. 5) and listed in Table 2. To obtain the kinetic parameters of ACPase II, one unit of enzyme activity was defined as 1 μ mol of inorganic phosphate release from pNPP or phosphorylated saccharides per min. The kinetic parameters for 3²-phosphoryl maltotriose, 6²-phosphoryl maltotriose, and PO-1 were calculated by using molecular weights of 3²-phosphoryl maltotriose and 6²-phosphoryl maltotriose (both 582.4, as free acid form), and PO-1 fraction (estimated to be average 750). The K_m of ACPase II for 3²-phosphoryl maltotriose was almost the same as that for 6²-phosphoryl maltotriose. However, the k_{cat} for 3²-phosphoryl maltotriose was about one-third of that for 6²-phosphoryl maltotriose.

A. niger KU-8 produced another type of intracellular acid phosphatase, ACPase I. ACPase I was a major intracellular acid phosphatase in the strain KU-8. The K_m of ACPase I in partial purified form for pNPP, G1P and G6P were measured (Table 2), because the ACPase I showed little activity on PO-1 fraction including 3²-phosphoryl maltotriose and 6²-phosphoryl maltotriose. K_m value of ACPase I for pNPP was much lower than that of ACPase II. Since the molecular weight of native ACPase I could not be measured, the k_{cat} of ACPase I for each substrate was not calculated.

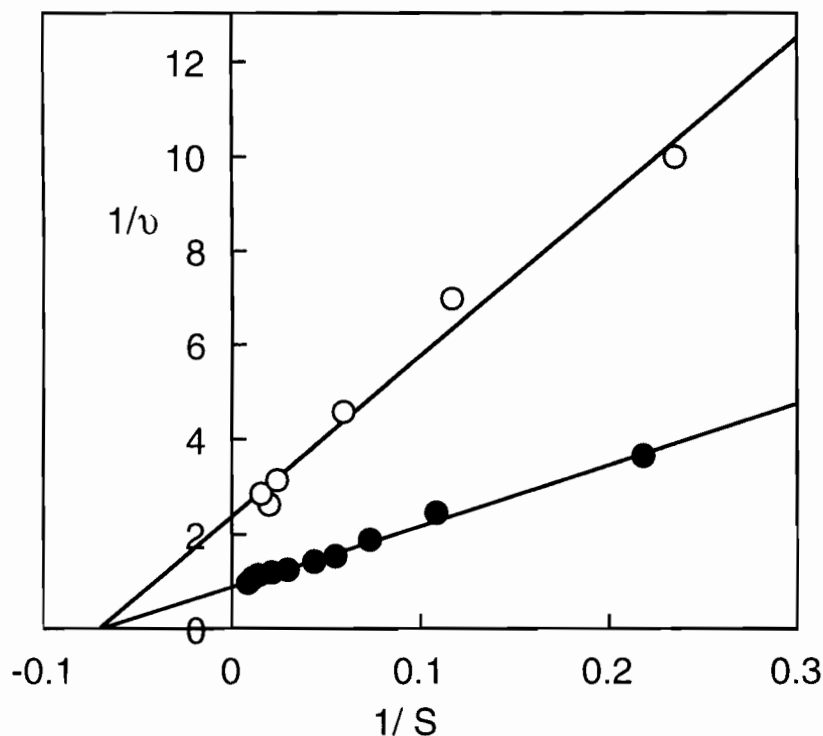


Fig. 5. Lineweaver-Burk Plots of ACPase II on 3²-Phosphoryl Maltotriose (○) and 6²-Phosphoryl Maltotriose (●).

Fifty μL of the enzyme (fixed at 0.01 unit, measured by p-nitrophenol release from pNPP) was incubated with various concentrations of each substrate (125 μL) for 2, 5, 10, and 15 min at 37°C in 0.2 M glycine-HCl (pH 2.0) buffer (325 μL). Initial velocities were calculated by measuring the release of inorganic phosphate from each substrate.

v , initial velocity for hydrolysis of substrate (mM product per min per mg protein);
 S , substrate concentration (mM).

Table 2. Michaelis Constant (K_m) and Molecular Activity (k_{cat}) of ACPase II

Substrates	K_m (mM)	k_{cat} (sec ⁻¹)	k_{cat}/K_m
pNPP	1.09 (0.34)*	5.72	5.25
Glucose 1-phosphate	2.00 (3.95)*	3.34	1.67
Glucose 6-phosphate	1.76 (4.99)*	5.20	2.95
PO-1	21.2	8.10	0.38
3 ² -phosphoryl maltotriose	14.1	1.82	0.13
6 ² -phosphoryl maltotriose	14.5	4.85	0.34

*Values in parenthesis are K_m of ACPase I.

2.4 Discussion

When reacted with the PO-1 fraction, ACPase II dephosphorylated all of the C-6 phosphate groups of 6-phosphoryl oligosaccharides irrespective of their DPs, as demonstrated in Chapter I, section 1. Therefore, it would be reasonable that the dephosphorylation rates of ACPase II toward 6²-phosphoryl maltotriose and other 6-phosphoryl oligosaccharides are almost the same. In 3-phosphoryl oligosaccharides, the activity of ACPase II toward 3³-phosphoryl maltotetraose was also almost the same for 3⁴-phosphoryl maltopentaose as the case of 6-phosphoryl oligosaccharides, although its dephosphorylation rate of C-3 phosphate groups was much slower than that of C-6. Therefore, the ACPase II activity toward 3-phosphoryl oligosaccharides (3³-phosphoryl maltotetraose and 3⁴-phosphoryl maltopentaose) would also be similar to 3²-phosphoryl maltotriose.

With regard to other substrates, the K_m of ACPase II for pNPP was higher than that of other acid phosphatases. For example, 0.27 mM with *Aspergillus ficuum* (Ullah and Cummins, 1987), 0.28 mM with yellow lupin (Olczak, *et al.*, 1997), 0.27 mM (at pH 3.8) with human prostate (Luchter-Wasylewska, 1996), and 1.0 mM with *A. niger* (Shimada *et al.*, 1974). Glucose 6-phosphate was a slightly better substrate of ACPase II than G1P.

A. ficuum NRRL 3135 extracellular pH 2.5 optimum acid phosphatase had the active site residues, RHGXRX_P (Ullah and Dischinger, 1993), which is homologous to acid phosphatases from *Saccharomyces cerevisiae* (Bajwa, *et al.*, 1984), *Escherichia coli* (Touati and Danchin, 1987), *A. niger* (MacRae, *et al.*, 1988) and human (Vihko, *et al.*, 1988). Of the amino acid sequence motif, RHG is highly conserved tripeptide. The substrate specificity (Wyss *et al.*, 1997b) and crystal structure (Kostrewa, *et al.*, 1999)

of *A. niger* NW 205 extracellular pH 2.5 optimum acid phosphatase (AnigAP) was described, they belongs to the family of high molecular weight acid phosphatases. The analysis of crystal structure identified that the active site of AnigAP was Arg62, His63 and Arg66, all from the RHGXRXP motif. The property of the AnigAP is very similar to the ACPase II as follows (AnigAP vs. ACPase II): optimum pH (2.5 vs. 2.0), molecular weights of monomer (65 vs. 66 kDa) and native form (tetramer, 269 vs. 260 kDa), and broad substrate specificity but no activity on phytic acid, with one except bion of the different localization (extracellular vs. intracellular). If ACPase II and AnigAP are similar or identical, the same active site residues as mentioned above may be conserved in ACPase II.

From the physiological viewpoint, whether the difference of the binding position of phosphate groups affects the function of POs is not clear so far. The characterization of ACPase II and 3-phosphoryl oligosaccharides may help to understand the characteristics of other acid and alkaline phosphatases and phosphate groups existing in various starches.

In diabetic complications, C-3 phosphorylated saccharides, sorbitol 3-phosphate and fructose 3-phosphate, have been detected by ^{31}P -NMR in diabetic in human erythrocyte and in mammalian lens as diabetes-associated metabolites (Szwergold *et al.*, 1989, 1990, Petersen *et al.*, 1990, 1992).

Thus, 3-phosphoryl oligosaccharides or its single component are thought to be very useful for better understanding of the biological and physiological roles of C-3 phosphorylated saccharides in diabetic complications.

2.5 Summary

The intracellular acid phosphatase II (ACPase II) produced by *Aspergillus niger* KU-8 preferentially dephosphorylates C-6 phosphate groups rather than C-3 phosphate groups of phosphoryl oligosaccharides. In this study, the kinetic parameters of ACPase II were measured. 3²-phosphoryl maltotriose and 6²-phosphoryl maltotriose, which differ only in the binding position of the phosphate group, were prepared and used as the substrates. The K_m for both substrates were similar. However, the k_{cat} value for the 6²-phosphoryl maltotriose was about three-fold of that for the 3²-phosphoryl maltotriose.

CHAPTER II

Bioavailability of phosphoryl oligosaccharides of calcium (POs-Ca) in rats

SECTION 1

In vitro solubility and *in vivo* digestibility of POs-Ca

1.1 Introduction

Phosphoryl oligosaccharides (POs) from potato starch hydrolysates have an inhibitory effect on the formation of a calcium phosphate precipitate (Kamasaka *et al.*, 1995). Moreover, calcium-bound POs (phosphoryl oligosaccharides of calcium; POs-Ca) would be a good foodstuff as soluble calcium material, because POs-Ca themselves are very soluble in water.

Uchida *et al.* (1996) previously reported the *in vitro* digestibility of POs using rat intestinal acetone powder. In the digestive tract, saccharides are digested by secretory and membrane enzymes on the surface of the intestinal mucosa, and then those are absorbed immediately as monosaccharides (Goda and Oku, 2002). Therefore, an *in vitro* system is insufficient to evaluate the digestion and absorption of saccharides, since there is no process by which the digests are absorbed.

Although the relationship between the solubility and bioavailability of calcium remains unclear (Heaney *et al.*, 1990), it is obvious that calcium must be ionized and in solution to be absorbed (Bronner *et al.*, 1986, Bronner, 1988, Pansu *et al.*, 1989).

In this section, the author first investigated the solubility of POs-Ca as a

calcium material for foods in comparison with the soluble calcium sources, calcium chloride and calcium lactate, and with the insoluble calcium sources, calcium carbonate and dibasic calcium phosphate. In addition, the author investigated the digestibility of POs-Ca by artificial gastric acid and by rat plasma glucose response after oral administration of POs-Ca.

1.2 Materials and Methods

1.2.1 Materials

Somogyi copper solution and Nelson solution were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals and materials were of analytical or commercial grade.

1.2.2 Preparation of POs and POs-Ca

POs was prepared from potato starch hydrolysate as described in Kamasaka *et al.* (1995), and then POs was desalted by cation-exchange resin (DOWEX 50W x 4, 20-50 mesh). Phosphoryl oligosaccharides of calcium (POs-Ca) were prepared by neutralizing the desalted POs solution (pH 2.0) with $\text{Ca}(\text{OH})_2$ to pH 6.5. POs-Ca was then freeze-dried. The calcium, phosphate and saccharide contents of POs-Ca were 5.0, 3.7 and 77.6 g / 100 g, respectively.

1.2.3 Comparison of *in vitro* calcium solubility

The solubility of each calcium compound was measured by the method of Tsugawa *et al.* (1999) with a slight modification. Each calcium compound (25 mg calcium /mL) was mixed into the solutions at 30°C and kept for 30 min while vigorously shaking for 30 s each time at 5-min interval. The excess amount of each calcium compound was removed by

filtration with 0.45 μm membrane and the calcium concentration of the filtrate was measured by the *o*-cresolphthalein complexon (OCPC) method with the Calcium C-test (Wako Pure Chemical Industries). The following solutions were used for the solubility test; distilled water, 0.1 M acetate buffer (pH 4.5), 0.1 M phosphate buffer (pH 4.5), 0.1 M HEPES buffer (pH 7.4), 0.1 M phosphate buffer (pH 7.4), 0.1 M carbonate buffer (pH 10.0) and 0.1 M phosphate buffer (pH 10.0).

1.2.4 Digestibility of POs-Ca by artificial gastric acid

The digestibility of POs-Ca by artificial gastric acid was studied by a modified method of Kaneko *et al.* (1995). The reaction mixture (1.0 mL) containing 50 mg/mL POs-Ca or sucrose in 0.125 M KCl-HCl buffer (pH 1.0, 1.5 and 2.0) were incubated at 37°C for 0, 1, 2 and 4 h. The reaction was terminated by neutralizing the mixture with 1 N NaOH. The increase of reducing sugar was measured by the Somogyi-Nelson method (Nelson, 1944, Somogyi, 1952). In the POs-Ca mixture, the concentration of inorganic phosphate was measured by the method of Itaya and Ui (1966) as described in Chapter I, section 1.

1.2.5 Effect of POs-Ca administration on plasma glucose response in rats

1.2.5.1 Animals

All rats were maintained according to the guideline for the care and use of laboratory animals of Ezaki Glico Co., Ltd. Twelve male Wistar rats (Japan SLC, Shizuoka, Japan) weighing 240-260 g were housed in individual wire cages in a temperature-controlled ($23\pm 2^\circ\text{C}$) room with $55\pm 7\%$ relative humidity and a 12-h light-dark cycle, with free access to tap

water and standard MF diet (Oriental Yeast, Tokyo, Japan). Rats were divided into three groups (n=4) after being acclimatized.

1.2.5.2 Administration of glucose, high maltotetraose syrup or POs-Ca to rats

The day before the experiment at 1700 h, food was withheld. The next day, 2 mL of 257 mg/mL POs-Ca solution (200 mg/mL solution as saccharides) was administered orally by direct stomach intubation for one group. Two mL of 200 mg/mL solution of glucose or high maltotetraose syrup (TETRUP[®]-H, Hayashibara Biochemical Laboratories Inc., Okayama, Japan) was administered to the remaining two groups as control saccharides. Before and after administration, blood samples were collected from the tail vein at each time point into heparinized capillary hematocrit tubes (Drummond Scientific Co., USA), and plasma was prepared by centrifugation at 11,000 rpm x 5 min (TH-1 rotor, TOMY SEIKO, Japan) at room temperature and stored at -40°C until assayed.

1.2.6 Measurement of plasma glucose, calcium and phosphate concentration

Concentrations of rat plasma glucose, calcium, and phosphate were measured using Glucose CII-test, Calcium C-test, and Phosphor C-test (Wako Pure Chemical Industries), respectively.

1.2.7 Statistical analysis

Results were subjected to one-way analysis of variance with Fisher's PLSD post hoc test. Differences in mean values between the groups were considered significant at $p < 0.05$ or 0.01 .

1.3 Results

1.3.1 Comparison of calcium solubility

The solubility of each calcium compound in distilled water and several buffer solutions are shown in Table 1. POs-Ca showed high solubility like that of calcium chloride in the absence or presence of phosphate in a wide pH range. The solubility of calcium lactate, which is also a soluble calcium compound, was about one-third that of POs-Ca. Calcium carbonate was relatively highly soluble in the acetate buffer (pH 4.5) among the test solutions, but was hardly dissolved in the other buffers, like dibasic calcium phosphate. The solubility of dibasic calcium phosphate was extremely low (0.39 mg calcium /mL) even in the acetate buffer (pH 4.5).

Table 1. Solubility of Calcium Compounds in Distilled Water or 0.1 M Buffer of pH 4.5, 7.4 and 10.0 at 30°C

Compound	Solubility (mg calcium / mL)							
	Distilled water	Buffer (0.1 M)						
		pH 4.5		pH 7.4		pH 10.0		
		Acetate	Phosphate	HEPES	Phosphate	Carbonate	Phosphate	
POs-Ca	23	23	23	23	21	22	21	
CaCl ₂	24	23	24	23	22	22	22	
Ca-lactate • 5H ₂ O	7.8	8.7	6.5	7.4	4.9	5.2	5.5	
CaCO ₃	<0.01	4.2	<0.01	0.2	<0.01	<0.01	<0.01	
CaHPO ₄ • 2H ₂ O	0.02	0.39	0.17	0.02	<0.01	0.02	<0.01	

The total calcium concentration of each solution was adjusted to 25 mg/mL.
Soluble calcium was measured by the OCPC method.

1.3.2 Digestibility of POs-Ca by artificial gastric acid

Figure 1 shows the digestibility of POs-Ca and sucrose by artificial gastric acid at pH 1.0, 1.5 and 2.0. The concentrations of inorganic phosphate in the POs-Ca mixture at each pH are shown in Table 2. A gradual and pH-dependent hydrolysis of sucrose was observed. On the other hand, no increases of reducing sugar and inorganic phosphate were observed in the POs-Ca mixture.

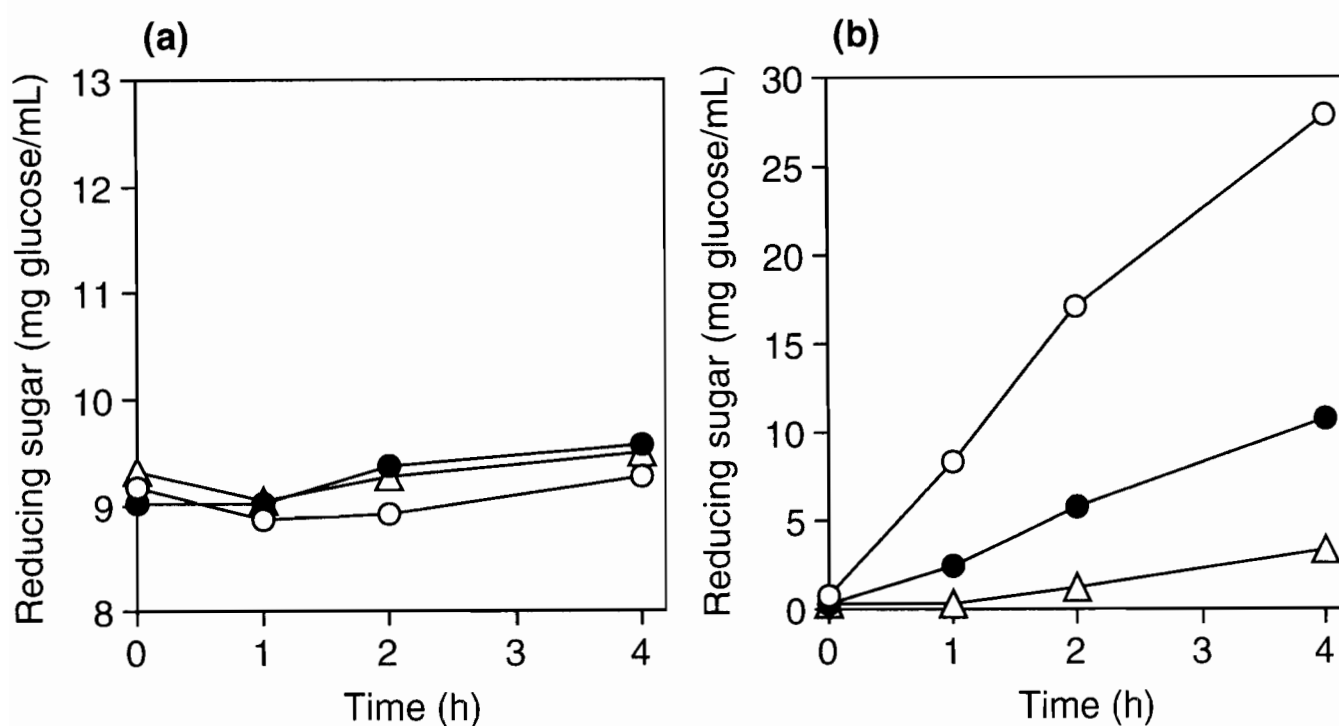


Fig. 1. Hydrolysis of POs-Ca (a) and Sucrose (b) by Artificial Gastric Acid.

The reaction mixture contained 50 mg/mL POs-Ca or sucrose. The reaction was performed at 37°C. ○, pH 1.0; ●, pH 1.5; △, pH 2.0. The reducing sugar in the mixture as measured by Somogyi-Nelson method. The data were represented as the means of triplicate measurements.

Table 2. The Concentration of Inorganic Phosphate (Pi) in the Mixture of POs-Ca and Artificial Gastric Acid

Incubation time (h)	pH		
	1.0	1.5	2.0
	Pi (mg/mL)		
0	0.033	0.033	0.033
1	0.033	0.033	0.033
2	0.032	0.033	0.032
4	0.033	0.033	0.033

The reaction mixture (1.0 mL) containing 50 mg/mL POs-Ca in 0.125 M KCl-HCl buffer (pH 1.0, 1.5 and 2.0) were incubated at 37°C.

Pi was measured by the method of Itaya and Ui (1966).

1.3.3 Effect of POs-Ca administration on plasma glucose response in rats

Figure 2 shows the changes of plasma glucose concentration when 400 mg of POs-Ca, glucose or high maltotetraose syrup (TETRUP-H) as saccharides were orally administered to rats. Although the glucose concentration of POs-Ca group was significantly lower than those of other groups at 0.25 h after administration, the overall rates of plasma glucose response in the three groups were almost similar. The maximum concentration of the POs-Ca group (179.02 ± 8.93 mg/dL at 0.5 h) was lower than those of the glucose group (205.98 ± 13.81 mg/dL at 0.25 h) and the TETRUP-H group (193.71 ± 29.47 mg/dL at 0.5 h), although the same amount of saccharides was administered.

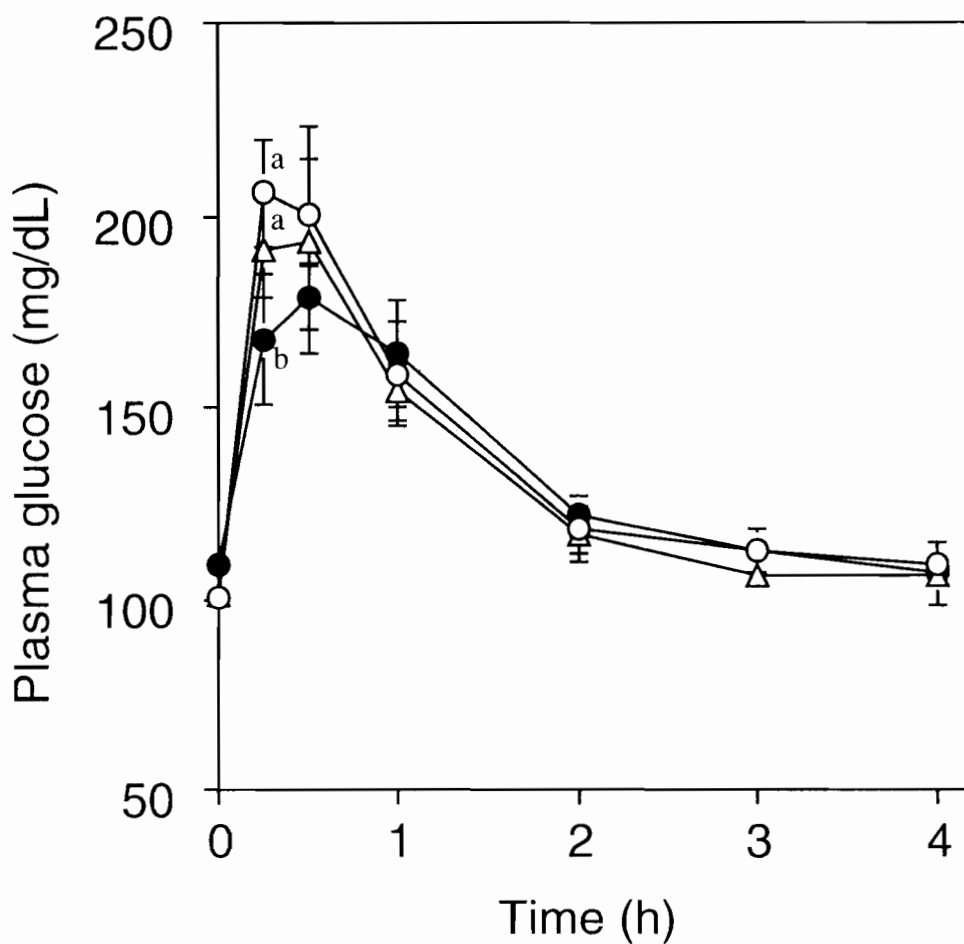


Fig. 2. Plasma Glucose Concentration in Rats after Administration of 400 mg POs-Ca, Glucose or High Maltotetraose Syrup (TETRUP-H).

Blood samples were collected from rat tail vein before and for 4 h after administration. Each point represents means $\pm$ SD for 4 rats. ^{a,b}Values not sharing a common superscript letters are significantly different ($p < 0.01$). ○, glucose; ●, POs-Ca; △, TETRUP-H.

1.3.4 Effect of POs-Ca administration on plasma calcium and phosphate in rats

Figure 3 shows the changes of calcium and phosphate concentration in plasma. Plasma calcium concentration of the POs-Ca group showed slight but significant increase during 0.25-0.5 h after administration concurrent with the increase of plasma glucose. On the other hand, the transient decrease during the rise of plasma glucose and the following recovery of plasma phosphate concentration was observed in all groups.

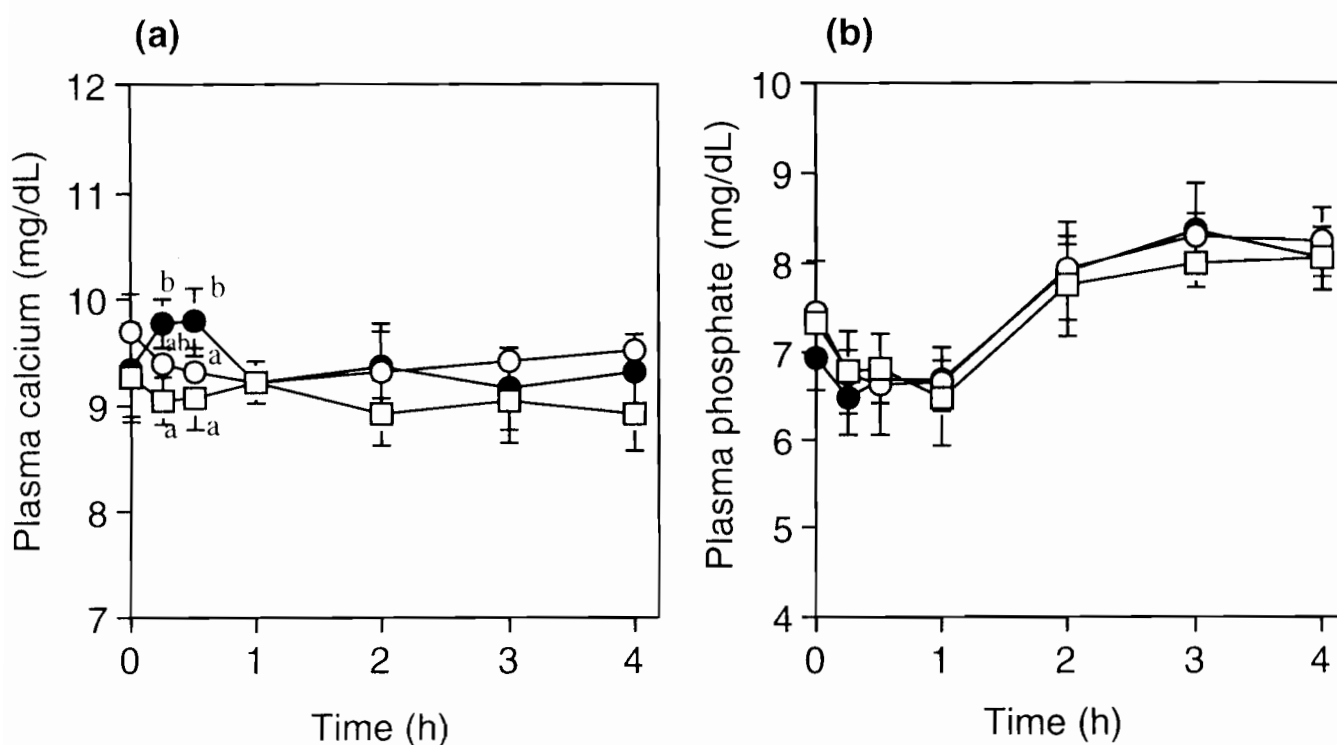


Fig. 3. Plasma Calcium (a) and Phosphate (b) Concentration in Rats after Administration of 400 mg POs-Ca, Glucose or High Maltotetraose Syrup (TETRUP-H).

Blood samples were collected from rat tail vein before and for 4 h after administration. Each point represents means $\pm$ SD for 4 rats. ^{a,b}Values not sharing a common superscript letters are significantly different ($p < 0.01$).

○, glucose; ●, POs-Ca; △, TETRUP-H.

1.4 Discussion

In this section, the author investigated the solubility of phosphoryl oligosaccharides of calcium (POs-Ca), digestibility of POs-Ca by artificial gastric acid and by rat plasma glucose response after oral administration of POs-Ca.

In the calcium solubility experiment, the author demonstrated that the solubility of POs-Ca was very high and comparable with that of calcium chloride over a wide pH range and even in the presence of phosphate. From this result, the author thought that most of the calcium from POs-Ca would remain soluble in the small intestine when POs-Ca is ingested by humans.

When POs-Ca was treated with artificial gastric acid of 0.125 M KCl-HCl buffer (pH 1.0, 1.5 and 2.0), the increase of reducing sugar and inorganic phosphate was not observed. From these results, the author thought that the α -1, 4-glucosyl bond and ester bond of phosphate groups of POs-Ca, especially C-3 phosphate group which is more instable than C-6, were not hydrolyzed by gastric acid.

When POs-Ca, glucose or high maltotetraose syrup as saccharides were orally administered to rats, the plasma glucose response in the three groups were almost similar. Because POs-Ca is only composed of glucose as saccharide, it will be reasonable that the changes of plasma glucose response after administration directly respond to the digestibility of POs-Ca. From the result, the author thought that the phosphate groups of POs-Ca did not influence the absorption, although POs-Ca is a mixture of phosphorylated oligosaccharides. Thus, it is thought that the dephosphorylation of POs-Ca by alkaline phosphatase localizing at the brush border membrane in the

small intestine would not be rate limiting in POs-Ca absorption. The result that the similarity of plasma glucose response between the glucose group and the TETRUP-H group agreed with the study of Wahlqvist *et al.* (1978) using glucose and a mixture of maltooligosaccharides (Caloreen, main DP = 5). Therefore, the author thought that the difference of DP of the maltooligosaccharides does not influence the rate of absorption of glucose significantly.

As to the digestibility of POs-Ca by human saliva α -amylase (HSA), Kamasaka *et al.* (1997c) reported the action of HSA on the PO-1 fraction. HSA could hydrolyze a part of the PO-1 fraction, but it took 24 h to hydrolyze POs. Consequently, the author thought that POs-Ca would not be substantially hydrolyzed by HSA when humans ingest POs-Ca in foods or drinks.

As to the changes of calcium and phosphate concentration in plasma when administered POs-Ca, the slight but significant increase of plasma calcium during 0.25-0.5 h after oral administration of POs-Ca was in accordance with the increase of plasma glucose. Thus, the author thought that the calcium increased was brought from POs-Ca. In addition, the author considered that the administration of large quantity of calcium from POs-Ca (about 25 mg) which corresponds to about one-third of daily calcium intake in rats (about 84 mg, Uchida *et al.*, 1996) resulted in the significant increase of plasma calcium. The relation between the increase in plasma glucose and decrease in plasma phosphate remains unclear so far.

In conclusion, the author considered that the *in vitro* solubility of POs-Ca was the same as that of calcium chloride, and that the POs-Ca orally administered would be hydrolyzed into glucose, calcium and phosphate and absorbed completely in the small intestine.

1.5 Summary

The solubility and bioavailability of phosphoryl oligosaccharides of calcium (POs-Ca) were investigated. The solubility of POs-Ca was as high as that of calcium chloride and about 3-fold higher than that of calcium lactate. POs-Ca was not hydrolyzed by artificial gastric acid. The overall rate of plasma glucose response after oral administration of POs-Ca to rats was similar to that in the case of administration of control saccharides, namely, glucose and high-maltotetraose syrup (TETRUP-H). However, the glucose concentration of POs-Ca group at 0.25 h after administration was significantly lower than those of other groups. In the case of POs-Ca administration, plasma calcium concentration showed slight but significant increase during 0.25-0.5 h concurrent with the increase of plasma glucose. On the other hand, plasma phosphate concentration decreased and then increased gradually from 2 h after the administration. From these results, it was suggested that the POs-Ca orally administered would be hydrolyzed into glucose, calcium, and phosphate and then absorbed completely in the small intestine, therefore not causing acute diarrhea.

SECTION 2

Calcium absorbability of POs-Ca in comparison with various calcium compounds and effect of POs-Ca feeding in rats

2.1 Introduction

Calcium is the major cation of bone minerals and plays an important role in mammalian metabolism (Bronner and Pansu, 1999). It is generally recognized that the absorbability of calcium is substantially dependent on its solubility in an aqueous solution (Pak and Avioli, 1988, Heaney *et al.*, 1990, Tsugawa *et al.*, 1999), because calcium will be absorbed in its free or ionized form in the intestine (Schachter *et al.*, 1960, Levenson and Bockman, 1994). A number of studies in recent years have reported on attempts to improve the bioavailability of calcium by the addition of such compounds as citric acid (Lacour *et al.*, 1997) casein phosphopeptides (CPP) (Hansen *et al.*, 1997), or by use of highly soluble salts such as calcium gluconate (Pansu *et al.*, 1993).

Phosphoryl oligosaccharides (POs) are prepared from enzymatic hydrolysate of potato starch. Phosphoryl oligosaccharides of calcium (POs-Ca) form a complex consisting of POs and calcium. POs-Ca contains about 5% calcium and can be expected to be a useful calcium supplement because of its high solubility in water.

In this section, first, by using *in situ* rat ligated jejunum loop system, the author investigated the absorbability of calcium from POs-Ca by comparing with the soluble calcium sources, calcium chloride and calcium lactate, and with the insoluble calcium sources, calcium carbonate and dibasic calcium phosphate. Second, the author investigated whether the

total calcium absorption would be increased at the ligated loop when mixed POs-Ca and a whey mineral complex, which is widely used as a calcium source in various foods or drinks, were substituted for POs-Ca in the test solution. Third, the author investigated the effect of POs-Ca feeding on growth and calcium absorption in rats.

2.2 Materials and Methods

2.2.1 Chemicals

Calcium chloride, calcium lactate, calcium carbonate and dibasic calcium phosphate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Whey mineral complex (WMC, 22% calcium content) was obtained from Kyodo Milk Industry Co. (Tokyo, Japan). All other chemicals and materials were of analytical or commercial grade.

2.2.2 Comparison of *in situ* calcium absorbability using rat ligated jejunum loop

2.2.2.1 Animals

All rats were maintained according to the guideline for the care and use of laboratory animals of Ezaki Glico Co., Ltd. Male Wistar rats (Japan SLC, Shizuoka, Japan) weighing 240-260 g were housed in individual wire cages in a temperature-controlled ($23 \pm 2^{\circ}\text{C}$) room with $55 \pm 7\%$ humidity and a 12-h light-dark cycle, with free access to deionized water and a standard MF diet (Oriental Yeast, Tokyo, Japan).

2.2.2.2 Time-course characteristics of calcium absorption from POs-Ca

Calcium absorption from POs-Ca was measured by changing the time after injecting the POs-Ca solution into the ligated jejunum loop. Twenty-four rats were divided into six groups ($n=4/\text{group}$) after being

acclimatized. After a longitudinal abdominal incision under anesthesia with pentobarbital, the contents remaining in the upper intestine were washed out with saline (9 g/L NaCl solution) according to the method of Gunshin *et al.* (1989). The intestine was then ligated approximately 5 cm and 15 cm distal to the end of the ligament of Treitz, and 0.8 mL of a 12 mg/mL POs-Ca solution (0.6 mg/mL as calcium) was injected into the ligated loop. The loops of one group were immediately removed as the zero time control, and those of the remaining five groups were removed 0.25, 0.5, 1, 2 or 3 h after injection.

The contents of each loop were flushed out with 5.0 mL of 0.05 N HCl, and each sample was filled up to 6.0 mL with 0.05 N HCl. After filtration with Ultrafree-MC (nominal molecular weight limit of 30,000, Millipore), the amount of calcium in a flushed-out sample was measured by the OCPC method using Calcium C-test (Wako Pure Chemical Industries). The calcium absorption (%) was calculated as follows:

$$\text{Calcium absorption (\%)} = \{(A - B) / A\} \times 100$$

A is the average calcium concentration (mg/mL) in the loop of the control rats which had been removed at zero time, and B is the residual calcium concentration (mg/mL) in the loop of rats at each time.

The amount of saccharides in the loop was measured by the phenol-sulfuric acid method (Dubois *et al.*, 1989). In addition, the residual saccharides in the loop were analyzed by thin-layer chromatography (TLC). Ten μ L of the flushed-out samples was spotted on a Merck Kiesel gel 60 plate. TLC was carried out by the ascending method with a solvent system of ethanol : water : acetic acid (= 35:15:1, v/v). Spots

were visualized by spraying with H₂SO₄-methanol (1:1, v/v) followed by heating at 130°C.

2.2.2.3 Comparison of calcium absorbability

The composition of each test solution (0.8 mg/mL as calcium) is given in Table 1. Each test solution, except for POs-Ca group, contained 1.24% of high-maltotetraose syrup (TETRUP-H, Hayashibara Biochemical Laboratories Inc., Okayama, Japan), since the test solution of the POs-Ca group contained 1.24% of maltooligosaccharides.

In both the comparative experiments, thirty-six rats were divided into three groups (n=12/group) after having been acclimatized. The method of loop preparation and the injection volume of each test solution was the same as described above. For each group, the ligated loops of 4 rats were immediately removed as the zero time control (control rats), and those of the remaining 8 rats were removed 2 h after the injection (test rats). The calcium absorption (%) was calculated in the same way as described above.

Table 1. Composition of Each Test Solution for the Ligated Jejunum Loop Study

Ingredient	Group				
	POs-Ca	CaCl ₂	Ca-lactate	CaCO ₃	CaHPO ₄
POs-Ca (mg)	160	–	–	–	–
CaCl ₂ (mg)	–	22	–	–	–
Ca-lactate • 5H ₂ O (mg)	–	–	62	–	–
CaCO ₃ (mg)	–	–	–	20	–
CaHPO ₄ • 2H ₂ O (mg)	–	–	–	–	34
1 M HEPHS buffer (pH7.4) (mL)	0.3	0.3	0.3	0.3	0.3
200 mg/mL TETRUP-H (mL)	–	0.62	0.62	0.62	0.62
Distilled water (mL)	up to 10 mL				

The total calcium concentration of each solution was adjusted to 0.8 mg/mL.

2.2.3 Comparison of calcium absorbability with the combined use of a whey mineral complex (WMC) and POs-Ca using rat ligated jejunum loop

The composition of each solution is given in Table 2. The total calcium content of each test solution was fixed at 1.2 mg/mL, and the ratio of calcium from WMC to POs-Ca in the test solution was adjusted to that of WMC alone (1.2:0 mg/mL in the test solution), 2:1 (0.8:0.4 mg/mL) or 1:1 (0.6:0.6 mg/mL). Thirty-six rats were divided into three groups (n=12/group) after having been acclimatized. The numbers of the control (n=4) and test rats (n=8) in each group and the experimental method were the same as those described in 2.2.2.3 in Chapter II, section 2.

Table 2. Composition of Each Test Solution for the Combination of POs-Ca with the Whey Mineral Complex (WMC)

Ingredient	Group		
	WMC	2:1*	1:1*
POs-Ca (mg)	-	80	120
WMC (mg)	55	36	27
200 mg/mL TETRUP-H (mL)	0.9	0.6	0.5
Distilled water (mL)	up to 10 mL		

The total calcium concentration of each solution was adjusted to 1.2 mg/mL.

* Calcium ratio of the WMC to POs-Ca.

2.2.4 Effect of POs-Ca on rat growth and calcium absorption

2.2.4.1 Animals and diets

All rats were maintained according to the guideline for the care and use of laboratory animals of Ezaki Glico Co., Ltd. Twenty male Wistar rats (Japan SLC, Shizuoka, Japan) weighing 280-300 g were housed in individual wire cages in a temperature-controlled ($23\pm 2^{\circ}\text{C}$) room with $55\pm 7\%$ relative humidity and a 12-h light-dark cycle, with free access to deionized water and standard MF diet (Oriental Yeast, Tokyo, Japan). After being acclimatized, rats were divided into two groups ($n=10$).

The composition of the experimental diet was shown in Table 3.

Table 3. Composition of Experimental Diets

Ingredient	Control	POs-Ca
	g/100g	
Casein	20.0	20.0
DL-Methionine	0.3	0.3
α -Corn starch	63.9	55.4
Corn oil	5.0	5.0
Mineral mix*	1.6	1.6
Vitamin mix**	1.0	1.0
Cellulose	5.0	5.0
Choline bitartrate	0.4	0.4
POs-Ca	-	10.0
Tripotassium citrate monohydrate	-	1.3
CaCO_3	1.2	-
KH_2PO_4	1.6	-
Calcium		0.5
Phosphorus		0.4

*Mineral mixture (calcium and phosphorus free) was prepared by subtracting CaCO_3 and KH_2PO_4 from AIN-93G mineral mixture (Reeves *et al.*, 1993). **AIN-93G vitamin mixture.

This diet was based on the AIN-93G formula (Reeves *et al.*, 1993). The POs-Ca diet contained 10% POs-Ca, replacing α -corn starch in the control diet. To adjust the calcium and phosphorus contents, CaCO_3 and KH_2PO_4 were used in the control diet. Each group was given deionized water and the diet for 15 days. The body weight and food intake were recorded every day.

2.2.4.2 Calcium balance study

To measure the apparent calcium absorption, feces and urine were collected for the last 5 days. At the end of the experiment, the rats were anesthetized with pentobarbital and blood samples were collected from the abdominal aorta. The serum was prepared by centrifugation (3,000 g x 10 min) and stored at -40°C until assayed. The liver and cecum were removed and weighed.

2.2.4.3 Analysis of serum

The serum calcium, phosphate, triglyceride, phospholipid and total cholesterol were measured using Calcium C-test, Phosphor C-test, Triglyceride G-test, Phospholipid B-test and Cholesterol C-test, respectively (Wako Pure Chemical Industries).

2.2.4.4 Analysis of feces and urine

The amounts of calcium were determined by inductively coupled plasma atomic emission spectrometry (ICP, SPS7700, Seiko Instruments Inc.). All feces were dried at 95°C for 6 h and then powdered and homogenized by mill-mixer. An aliquot (1.0 g) of each powdered feces were ashed at 550°C for 16 h in a muffle furnace. The ashed samples were dissolved

with 5.0 mL of 10% HCl and diluted with distilled water and filtrated by 0.45 µm membrane, and then subjected to ICP. Urine samples were diluted with distilled water and filtrated by 0.45 µm membrane, and then directly subjected to ICP. The apparent calcium absorption ratio and retention ratio were determined by the following equations:

$$\text{Absorption ratio (\%)} = (\text{intake} - \text{fecal excretion}) / (\text{intake}) \times 100$$

$$\text{Retention ratio (\%)} = (\text{intake} - \text{fecal excretion} - \text{urinary excretion}) / (\text{intake}) \times 100$$

2.2.5 Statistical analysis

Results were subjected to one-way analysis of variance with Fisher's PLSD post hoc test. Differences in mean values between the groups were considered significant at $p < 0.05$.

2.3 Results

2.3.1 Comparison of *in situ* calcium absorbability

2.3.1.1 Time-course characteristics of calcium absorption from POs-Ca

The time-course characteristics for calcium absorption from POs-Ca and residual POs-Ca in the ligated jejunum loop is shown in Fig. 1.

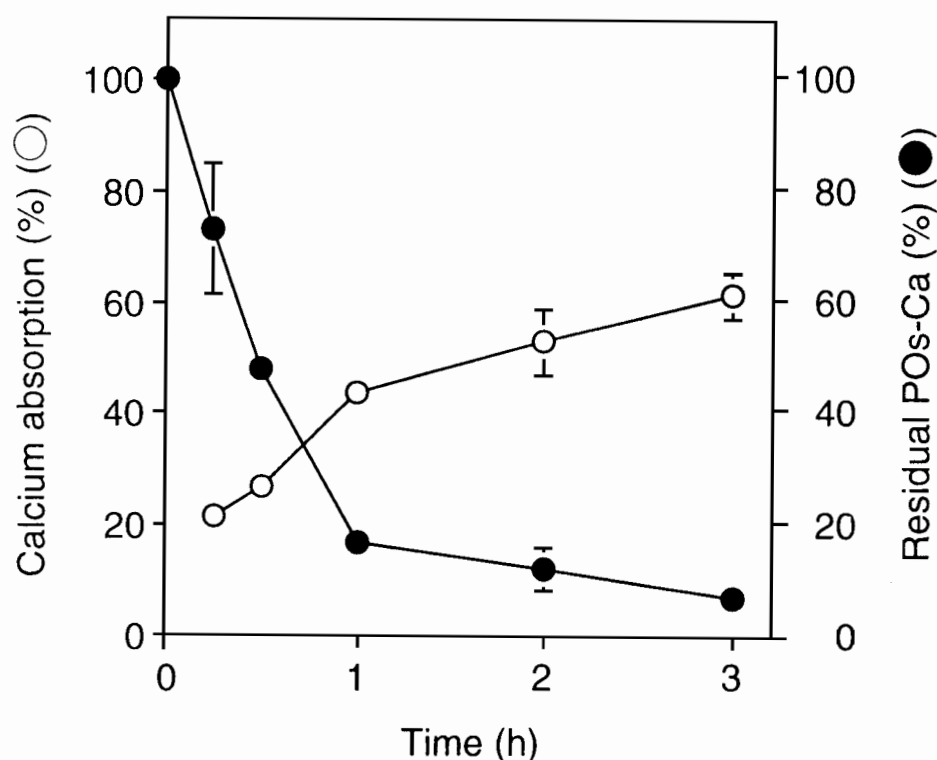


Fig. 1. Changes in the Calcium Absorption Rate (○) and the Amount of Residual POs-Ca in the Rat Ligated Jejunum Loop (●) after Injection of POs-Ca.

Each value is the mean $\pm$ SD for 4 rats. The amount of POs-Ca was measured by phenol-sulfuric acid method. The amount of the residual POs-Ca in the ligated loop at 0 time is designated as 100%.

The calcium absorption rate had almost reached a plateau 2 h after injecting the POs-Ca solution, so the loop was removed 2 h after injection of the test solution in subsequent experiments. The residual amount of POs-Ca in the loop time-dependently decreased, and less than 20% of POs-Ca was left in the loop 2 h after its injection. The disappearance of POs-Ca in the flushed-out samples was also confirmed in the TLC analysis (Fig. 2).

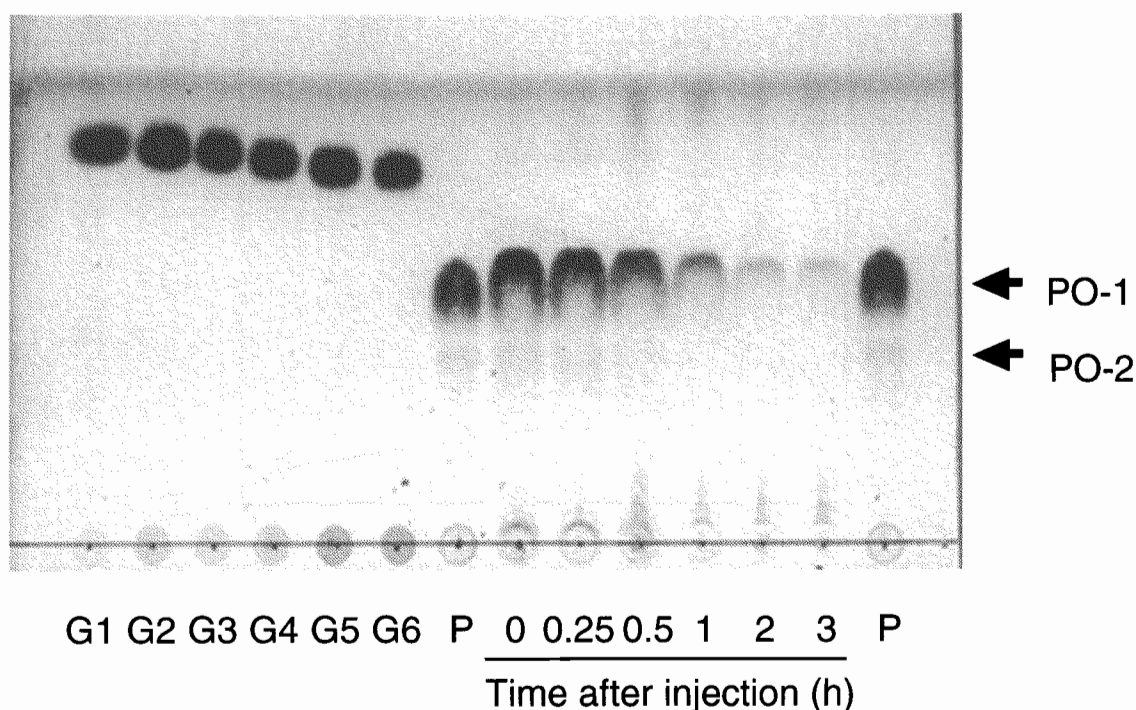


Fig. 2. Thin-Layer Chromatogram of the Saccharides in the Rat Ligated Jejunum Loop.

Ten μ L of samples flushed-out from the loop was spotted on a silica gel TLC plate. The solvent was ethanol:water:acetic acid (=35:15:1, v/v). G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose, G5, maltopentaose; G6, maltohexaose; P, POs-Ca.

2.3.1.2 Comparison of calcium absorbability from POs-Ca with various calcium compounds

A comparison of the calcium absorption rates in the ligated jejunum loop between POs-Ca and the soluble calcium compounds is shown in Fig. 3a. The calcium absorption rates of the three groups were all approximately 60%, so the intestinal calcium absorption rate of the POs-Ca group was almost comparable with that of the soluble calcium groups. However, as shown in Fig. 3b, the calcium absorption rate of POs-Ca was significantly higher ($p < 0.05$) than that of the insoluble calcium compounds.

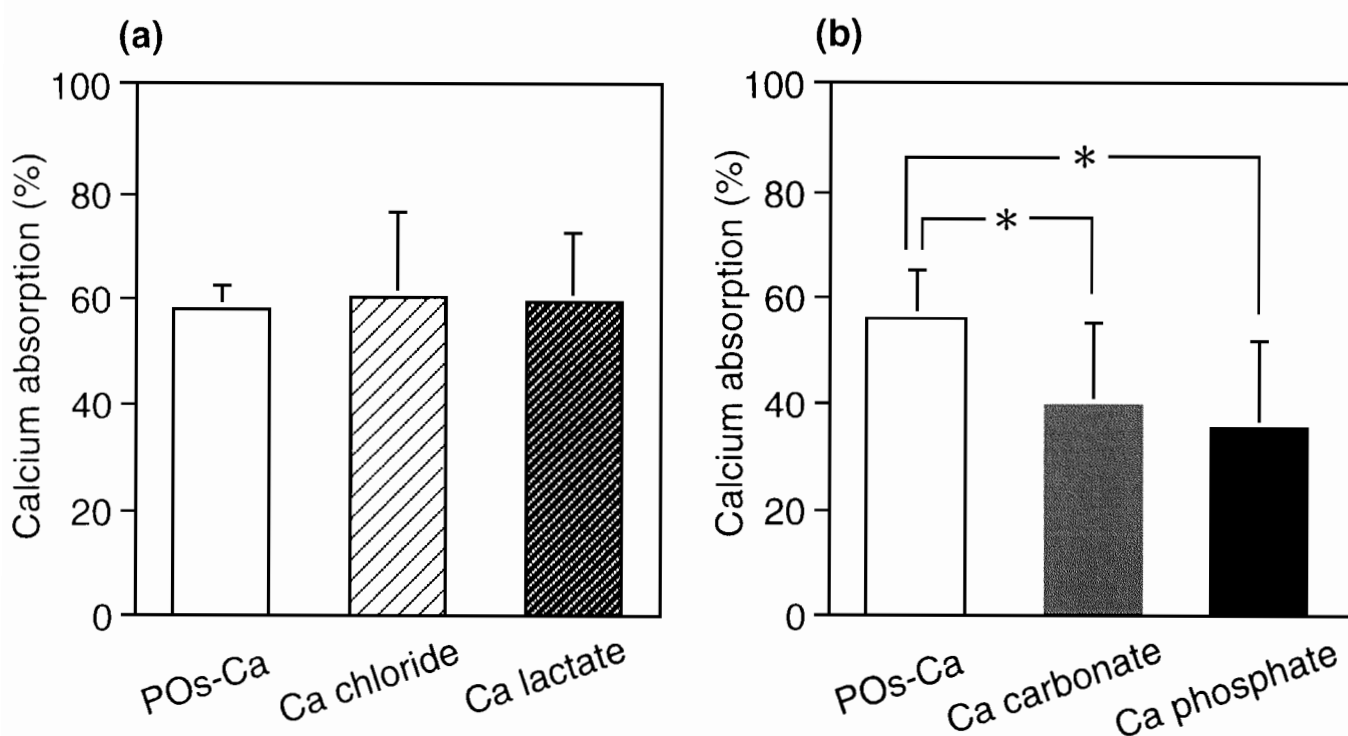


Fig. 3. Comparison of the Calcium Absorption of the POs-Ca Group with That of the Soluble Calcium Groups (a) and Insoluble Calcium Groups (b) in the Rat Ligate Jejunum Loop.

Each value is the mean $\pm$ SD for 8 rats. *Significantly different ($p < 0.05$) from the calcium carbonate and dibasic calcium phosphate groups.

The calcium solubility of the test solutions was measured by the OCPC method. As shown in Table 4, the calcium in the POs-Ca, calcium chloride and calcium lactate groups was reasonably soluble, but that in the calcium carbonate and dibasic calcium phosphate groups was insoluble.

Table 4. Soluble Calcium Concentration of the Test Solution for the Ligated Jejunum Loop Study

	Group				
	POs-Ca	CaCl ₂	Ca-lactate	CaCO ₃	CaHPO ₄
Soluble calcium (mg/mL)	0.77	0.86	0.74	0.11	0.016
%*	96.2	107.4	93.1	13.8	2.0

Soluble calcium was measured by the OCPC method.

* Percentage of soluble calcium to total calcium (0.8 mg/mL) in each test solution.

2.3.2 Comparison of calcium absorbability with the combined use of a whey mineral complex (WMC) and POs-Ca

When part of the whey mineral complex (WMC) was substituted for POs-Ca in the test solution, the total calcium absorption rate in the ligated jejunum loop increased with increasing of calcium from POs-Ca (Fig. 4). The calcium absorption rate of the group with a calcium ratio of 1:1 in the test solution was significantly higher ($p < 0.05$) than that of the WMC group. The percentage of soluble calcium in the test solution of the group with a calcium ratio of 1:1 was 48%, whereas that of the WMC group was as low as 4%.

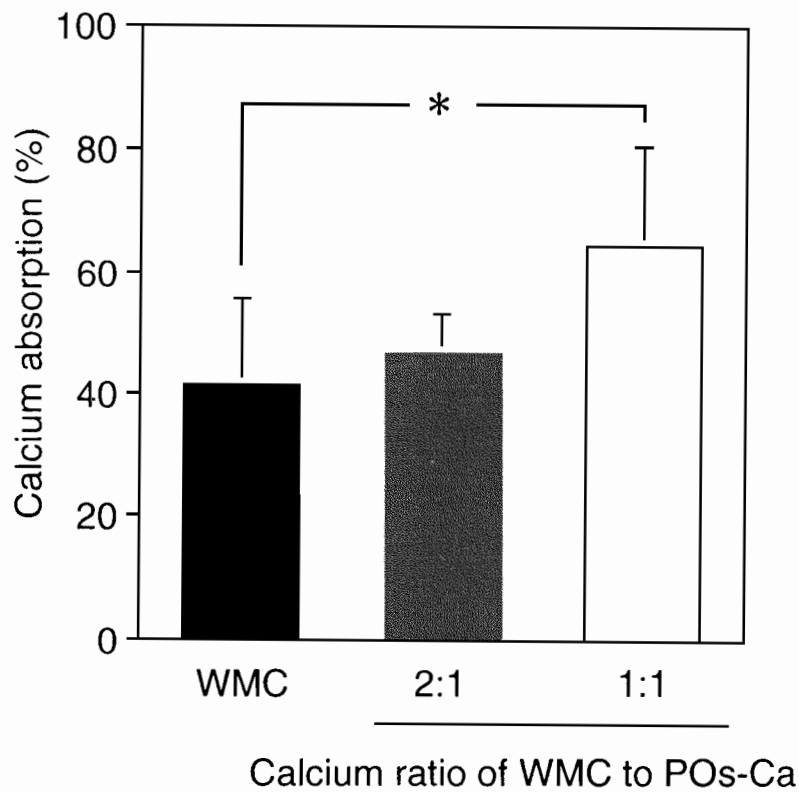


Fig. 4. Calcium Absorption in the Rat Ligate Jejunum Loop with Combined Use of the Whey Mineral Complex (WMC) and POs-Ca.

Each value is the mean $\pm$ SD for 8 rats. The total calcium concentration of each test solution was adjusted to 1.2 mg/mL by varying the calcium ratio of WMC to POs-Ca. *Significantly different ($p < 0.05$) from the WMC and 1:1 groups.

2.3.3 Effect of POs-Ca on rat growth and calcium absorption

2.3.3.1 Body weight and food intake, liver and cecum weight

Table 5 shows the body weight and food intake of the rats at the end of the feeding period. The final body weight and body weight gain of rats fed the diet containing 10% POs-Ca for 15 days were not significantly different from those of rats fed the control diet, and no diarrhea was observed in the POs-Ca group. Liver and cecum weight of the rats in the POs-Ca group also were not significantly different from those of the control group (Table 5).

Table 5. Effect of Feeding Diet Containing 10% POs-Ca on the Body weight, Food Intake and Organ Weight in Rats

	Control (n=10)	10% POs-Ca (n=10)
Initial body weight (g)	311.5±7.4	311.3±6.6
Final body weight (g)	379.0±12.5	374.4±10.9
Body weight gain (g)	67.5±11.6	63.2±6.8
Food intake (g/15day)	292.7±17.2	280.6±18.0
Food efficiency (gain/intake)	0.23±0.03	0.22±0.02
Liver weight (g)	3.35±0.33	3.15±0.25
Cecum weight (g)	1.31±0.38	1.50±0.25

Values are means ± SD for 10 rats.

2.3.3.2 Serum analysis

The concentrations of calcium, phosphate, triglyceride, phospholipids and total cholesterol in serum are shown in Table 6.

The triglyceride concentration of the POs-Ca group was significantly lower than that of the control group. No significant difference was observed

among the groups for the serum calcium, phosphate, phospholipid and total cholesterol concentrations.

Table 6. Effect of Feeding Diet Containing 10% POs-Ca on the Biochemical Indices in Rats

	Control (n=10)	10% POs-Ca (n=10)
Calcium (mg/dL)	10.57±0.79	10.04±0.44
Phosphate (mg/dL)	7.46±1.12	7.92±0.36
Triglyceride (mg/mL)	159.00±17.38	139.14±21.37*
Phospholipid (mg/dL)	155.10±9.46	156.41±8.00
Total cholesterol (mg/dL)	92.09±9.23	90.60±6.18

Values are means ± SD for 10 rats.

*Significantly different from the control group ($p<0.05$).

2.3.3.3 Calcium balance

Table 7 shows the apparent calcium absorption ratio and retention ratio during the balance study (10-15 days). No significant difference was observed among the groups for both the apparent calcium absorption and retention ratios. POs-Ca was not detected in the urine and feces (data not shown).

Table 7. Effect of Feeding Diet Containing 10% POs-Ca on the Apparent Calcium Absorption and Retention Ratio in Rats.

	Control (n=10)	10% POs-Ca (n=10)
Apparent absorption (%)	44.1±5.2	46.1±5.0
Apparent retention (%)	43.3±5.2	45.2±5.0

Values are means ± SD for 10 rats.

2.4 Discussion

In a previous section, the author described the solubility and digestibility of POs-Ca, suggesting that the POs-Ca is highly soluble and not hydrolyzed by gastric acid but completely hydrolyzed and absorbed in the small intestine. In this section, the author described the absorbability of POs-Ca as a calcium source by comparison with several calcium compounds and the effect of POs-Ca feeding on growth and calcium absorption in rats.

In the calcium absorbability experiment, the author demonstrated that the absorption of calcium from POs-Ca in the rat ligated jejunum loop was almost comparable to that from the soluble calcium compounds (calcium chloride and calcium lactate) and significantly higher than that from the insoluble calcium compounds (calcium carbonate and dibasic calcium phosphate). It is generally considered that calcium must be in a soluble and ionized form to be absorbed from the intestine. Therefore, the amount of calcium absorbed from a calcium compound would depend on its solubility. Although the relationship between calcium solubility and absorbability remains unclear, it is true that at least some of the orally ingested calcium would be dissolved in gastric acid. However, the absorption of calcium does not take place from the stomach, but from the small intestine, and mainly from the jejunum and ileum, where the pH value is commonly above 6.5 (Heaney *et al.*, 1990, Duflos *et al.*, 1995, Bronner *et al.*, 1999).

Intestinal calcium absorption comprises two processes; an active transcellular process that takes place in the duodenum and a passive paracellular process throughout the small intestine (Bronner, 1998, Bronner and Pansu, 1999). The active calcium transcellular process is saturable

and requires metabolic energy and dependent on vitamin D. The transcellular movement involves three steps; entry, intracellular diffusion, and extrusion. Transcellular calcium transport varies directly with the cellular content of calcium-binding protein (calbindin) which expression is regulated by vitamin D (Bronner *et al.*, 1986, Pansu *et al.*, 1989). The passive calcium transport, namely, paracellular nonsaturable diffusion, occurs down a chemical gradient, with calcium flux being directly proportional to luminal calcium concentration (Bronner and Pansu, 1999). Absorption of calcium is adjusted according to the level of plasma calcium concentration. When calcium intake is low, active transcellular calcium transport in the duodenum is upregulated and a large portion of calcium is absorbed by the active process than passive paracellular process. When calcium intake is adequate or high, passive absorption in the jejunum and ileum is the major (80 to 90% of the absorbed calcium, Pansu *et al.*, 1993) absorptive process.

There might be some hypoabsorption of calcium in the elderly because of the known increase in the prevalence of achlorhydria with age. The solubility and absorbability of calcium chloride were the same as those of POs-Ca, but calcium chloride is a relatively inferior calcium source because it irritates the gastrointestinal membrane (Tsugawa *et al.*, 1999). Recker (1985) has found no difference in calcium absorption between calcium citrate and calcium carbonate by normal subjects, but a large difference in patients with achlorhydria. Thus, the author considered that the high solubility of POs-Ca throughout the acidic, neutral and alkaline pH ranges would be advantageous for calcium absorption by the elderly and/or by patients with achlorhydria.

Partial substitution by POs-Ca for the whey mineral complex (WMC) in

the test solution elevated the total calcium absorption when the total calcium concentration of each test solution was equal to 1.2 mg/mL. It might be thought that the increase in the ratio of soluble calcium in the test solution resulted in the elevation of total calcium absorption. It is thus suggested that the increase in the ratio of soluble calcium in foods and in the intestinal tract would enhance the total calcium absorption. WMC is added as a calcium source to many kinds of processed food, especially to dairy products such as milk beverages and ice cream, due to its favorable flavor and image of being manufactured from milk, although its solubility is relatively low (about 4%). Thus, the author thought that the addition of POs-Ca to foods and drinks containing WMC or other insoluble calcium sources for the purpose of calcium fortification would be helpful to elevate the total absorption of calcium from them.

In the calcium balance study, the body weight and food intake of the rats fed the diet containing 10% POs-Ca for 15 day were not different from those of rats fed the control diet. In addition, it is important that no diarrhea was observed in the POs-Ca group through the feeding period, although the author could not demonstrate the significant increase in calcium absorption. Therefore, the author considers that POs-Ca would not cause acute diarrhea unlike other indigestible oligosaccharides, even by human ingestion by a large amount of POs-Ca at one time. On the other hand, the lowering effect of serum triglyceride concentration on POs-Ca was remains unclear in this study. Further works with respect to the effect of POs-Ca on calcium absorption and lipid metabolism are needed.

Phosphorylated guar gum hydrolysate (P-GGH) (Watanabe *et al.*, 2000), which was a different type of phosphorylated oligosaccharide, inhibited the

formation of a calcium-phosphate precipitate and increased the calcium absorption *in vivo*. Preparation of P-GGH needs chemical introduction of phosphate residues into a partial hydrolysate of guar gum. In contrast to P-GGH, POs-Ca can be easily prepared from the enzymatic hydrolysate of potato starch. Although the effect of POs-Ca on calcium absorption *in vivo* is not obvious at present, it is most likely that POs-Ca would increase the calcium absorption *in vivo* because POs-Ca also has phosphate groups in its structure, forming a soluble complex with calcium similar to P-GGH or casein phosphopeptide (CPP).

In conclusion, the author considered that the *in situ* Ca absorbability of POs-Ca was the same as that of calcium chloride and calcium lactate. Moreover, as a calcium supplement for various foods, especially for beverages, POs-Ca would be a favorable soluble calcium material with relatively high absorbability from the intestinal tract.

2.5 Summary

The absorbability of phosphoryl oligosaccharides of calcium (POs-Ca) as a calcium material was investigated by comparing with the soluble calcium compounds, calcium chloride and calcium lactate, or insoluble calcium compounds, calcium carbonate and dibasic calcium phosphate. An *in situ* experiment showed that the intestinal calcium absorption rate of POs-Ca was almost comparable with those of the soluble calcium compounds, but the absorption rate was significantly higher ($p < 0.05$) than those of the insoluble calcium groups. Moreover, the total absorption rate of a 1:1 mixture of the calcium from POs-Ca and a whey mineral complex (WMC) was significantly higher ($p < 0.05$) than that of WMC alone.

Body weight gain, food intake and food efficiency of rats fed the diet containing 10% POs-Ca for 15 days were not different from those of rats fed control diet, and no diarrhea was observed in the POs-Ca group. Apparent calcium absorption and retention between the groups were not significantly different. The serum triglyceride concentration of POs-Ca group was significantly lower than that of the control group. Other serum biochemical indices and organ (liver and cecum) weight were not significantly different between the groups. These results suggest that POs-Ca would be a useful soluble calcium material with relatively high absorption in the intestinal tract.

CHAPTER III

Remineralization effect of chewing gum containing phosphoryl oligosaccharides of calcium (POs-Ca) on enamel lesion in children *in vitro*

1. Introduction

Dental caries is an infectious disease resulting in destruction of tooth structure by acid forming bacteria found in dental plaque, and is a reversible multifactoral process (Steinberg, 2002, Hicks *et al.*, 2003 and 2004).

In recent years, Inaba *et al.* (2002a, 2002b, 2002c, 2003) and Kamasaka *et al.* (2002) demonstrated that the significant remineralization effect of sugar-free chewing gum containing 2.5% POs-Ca (POs-Ca (+) gum) on artificial early caries lesions in enamel *in vitro* and *in situ*. In those studies, the remineralization effect by the two types of the experiment was confirmed; the remineralization effect of the POs-Ca (+) gum by *in vitro* human saliva immersing (HSI) test (Kamasaka *et al.*, 2002) and by *in situ* intraoral study (Inaba *et al.*, 2002a).

The HSI-test makes it easy to standardize the experimental conditions and is easier task for volunteers than intraoral study. It is therefore thought that HSI-test could be an alternative method for the intraoral study. In both experiments, sugar-free gum without POs-Ca (POs-Ca (-) gum) was used as a control gum, and the remineralization effect was significantly higher in the POs-Ca (+) gum than that in POs-Ca (-) gum to a same extent. Thus, the result of HSI-test is very consistent with the intraoral study throughout of the all experiments, but all of those data has been obtained by using the adult volunteers. In this Chapter, the author investigated the remineralization effect of the POs-Ca (+) gum on children volunteers by *in vitro* HSI test.

2. Materials and Methods

2.1 Subjects

We studied 22 children volunteers (7-15 yr, 9 boys and 13 girls). The mean age of boys and girls were 11 and 10 years old, respectively. The procedures, possible benefits and disadvantages of the study were fully explained to the children's parents, and their informed consent was obtained prior to the study. All parents agreed to participate in the study. The study design was approved by the Human Investigations Review Committee of Ezaki Glico Co., Ltd.

2.2 Test gums

Two types of sugar-free chewing gum (tablet type) were used. One contains 46% xylitol and 2.5% POs-Ca (POs-Ca (+) gum), and the other contains 48.5% xylitol and no POs-Ca (POs-Ca (-) gum). The average weight of each gum was 1.5 g.

2.3 Preparation of demineralized bovine enamel slabs

Bovine incisors were used. Following removal of the roots, sound enamel slabs (approx. 7x7x3 mm) were cut from the crown using a water-cooled diamond saw (LUXO, Japan). The enamel samples were embedded in an acrylic resin (UNIFAST Trad, GC, Japan) except for the original enamel surface. The buccal enamel surfaces of these slabs were slightly abraded with wet abrasive paper (grit 800) to provide flat plates. To form the artificial early caries lesions, the slabs were demineralized in a 0.1 M lactic acid gel containing 6% carboxymethylcellulose (pH 5.0) for 3 weeks at 37°C. The gel volume was maintained at 100 mL per 6 enamels.

2.4 *In vitro* remineralization test by collected saliva

Each subject chewed one tablet of POs-Ca (+) gum or POs-Ca (-) gum

for 10 min and the whole saliva was collected into a plastic tube at each home. Demineralized bovine enamel slabs (2 samples per saliva sample) were immersed in the collected saliva for 40 min at 37°C. This procedure was done once a day and repeated for successive 4 days.

2.5 Quantification of mineral distribution

After the salivary treatments, planoparallel sections of about 500 μm thickness were cut from the enamel samples using a water-cooled diamond coated saw (Isomet, Buhler, USA). These sections were ground in a planoparallel direction with wet 800-grit abrasive paper to thickness of approximately 200 μm . The sections were fitted on a high resolution positive film (Fuji, Japan) and microradiographed (PW-1830, Phillips, Netherlands) by Cu-K α X-ray generated at 25 kV and 25 mA for 24 sec. The films were developed, fixed, and rinsed under standardized conditions. Digitized microradiographic images were analyzed for the lesion depth l_d (μm) by the combined means of computer-assisted videodensitometry (CAV) and a mineral distribution analysis (MDA) program developed by Inaba *et al.* (1997a, 1997b).

The definitions of l_d and mineral loss value ΔZ ($\text{vol}\% \cdot \mu\text{m}$) values and their schematical indications are shown in Table 1 and Fig. 1, respectively (Inaba *et al.*, 1997).

Table 1. Definitions of Microradiographical Mineral Parameters Associated with Dental Caries Lesions (See Fig. 1 for Schematical Indications)

Parameter and dimension	Definition
Sound mineral level V_s (vol%)	The mineral contents in vol% of the sound tissues being 89 vol% in enamel.
Outer surface position D_0	The border position where the mineral content is considered to change from zero to a positive value.
Lesion depth l_d (μm)	The distance from the outer surface position D_0 to the location in the lesion at which the mineral content reaches 95% level of the mineral content of the sound tissue.
Mineral loss value ΔZ (vol% $\cdot \mu\text{m}$)	Integrated value of the difference in the mineral vol% and the V_s value between the outer surface position D_0 and the lesion depth position.

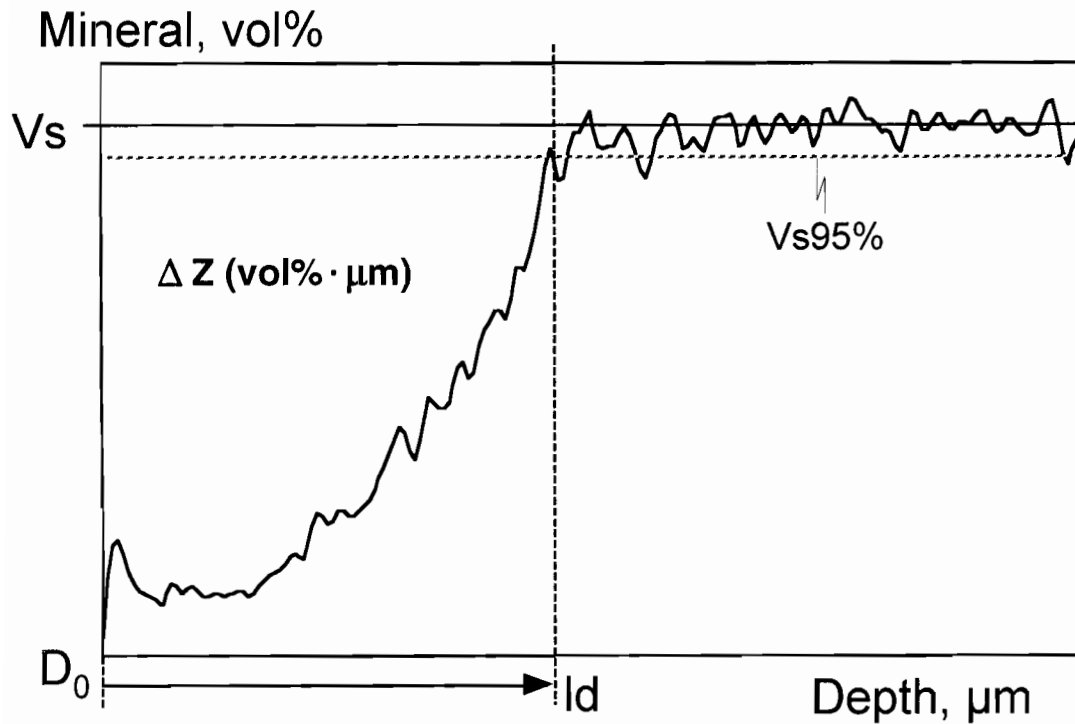


Fig. 1. Schematical Representation of the Mineral vol% Distribution of Demineralized Enamel with Related Main Mineral Parameters.

V_s , mineral vol% level of sound tissue; l_d , the lesion depth (μm); ΔZ , mineral loss value ($\text{vol\%} \cdot \mu\text{m}$).

2.6 Measurement of salivary volume, pH, calcium and phosphate

The saliva of each subject when chewing one tablet of gum (both type) for 10 min was newly collected to analyze the properties. The saliva was collected into a plastic tube and frozen at once at each home. After receiving the saliva samples, the volume of saliva was measured by reading the scale of the plastic tube directly, and the pH was measured using a portable pH meter. Then, an aliquot of saliva was centrifuged (10,000 rpm x 10 min). The supernatant was filtered with 0.45 µm membrane. The amount of calcium and phosphate in the filtrate were measured colorimetrically by *o*-cresolphthalein complexion (OCPC) method using Calcium C-test (Wako Pure Chemical Industries, Japan) and the method of Itaya *et al.* (1966), respectively.

2.7 Statistical analysis

The data were subjected to one-way analysis of variance with Tukey-Kramer post hoc test.

3. Results

3.1 Comparison of properties of the stimulated whole saliva

Properties of the saliva collected from the boys (n = 9) and girls (n = 13) when chewing one tablet of POs-Ca (+) gum or POs-Ca (-) gum for 10 min were shown in Table 2. The volume and pH of saliva stimulated by chewing gums was not significantly different in boys and girls, neither in both POs-Ca (+) gum and POs-Ca (-) gum groups.

Table 2. Comparison of Properties of Saliva Collected When Chewing Gum for 10 min between Boys and Girls

	Types of gum	Boys (n = 9)	Girls (n = 13)
Volume (mL)	POs-Ca (+)	11.72±5.59	10.65±4.98
	POs-Ca (-)	10.89±3.94	10.54±4.94
pH	POs-Ca (+)	7.87±0.35	7.95±0.27
	POs-Ca (-)	7.76±0.35	7.82±0.20
Ca (mM)	POs-Ca (+)	4.36±1.30*	4.93±1.00*
	POs-Ca (-)	1.54±0.29	1.59±0.32
P (mM)	POs-Ca (+)	5.70±1.02	5.58±0.91
	POs-Ca (-)	5.61±1.45	5.21±1.22
Ca/P ratio	POs-Ca (+)	0.76±0.18*, ^a	0.90±0.22*, ^b
	POs-Ca (-)	0.28±0.03	0.31±0.07

Values are means ± SD. *POs-Ca (+) vs. POs-Ca (-), ^{a,b}boys vs. girls.

Calcium concentration in the saliva was significantly higher in POs-Ca (+) gum group than that in POs-Ca (-) gum group for both boys and girls.

Phosphate concentration in the saliva was not significantly different in boys and girls in both gums groups.

Molar ratio of calcium/phosphate (Ca/P) in the saliva was significantly higher in POs-Ca (+) gum than in POs-Ca (-) gum for both boys and girls due to the significantly higher calcium concentration in the saliva, and that of the girls' saliva was significantly higher than that in boys saliva for the POs-Ca (+) gum.

3.2 Mineral distributions

Figure 2 shows comparison of the lesion depth (ld) and the mineral loss value (ΔZ) divided into the boy (n = 5) and girl (n = 8) volunteers in both types of gum. Although we recovered the saliva samples from all volunteers, boys (n = 9) and girls (n = 13), we could recover the enamel samples from 5 boys and 8 girls. In both boys and girls, the ld (means $\pm$ SD = 86 ± 27 μm for boys and 85 ± 15 μm for girls) in POs-Ca (+) gum were significantly lower than those in POs-Ca (-) gum (117 ± 16 μm for boys and 118 ± 12 μm for girls) and those after initial demineralization (Dem; 123 ± 11 μm , n = 10).

Figure 3 shows the comparison of ld and the ΔZ values between the types of gum. The ld value in POs-Ca (+) gum group (85 ± 19 μm) was significantly lower than that in POs-Ca (-) gum group (118 ± 13 μm). The ΔZ of all individual data in POs-Ca (+) gum were lower than that in POs-Ca (-) gum, but there was no significant difference between the two gum groups due to the relatively large standard deviations.

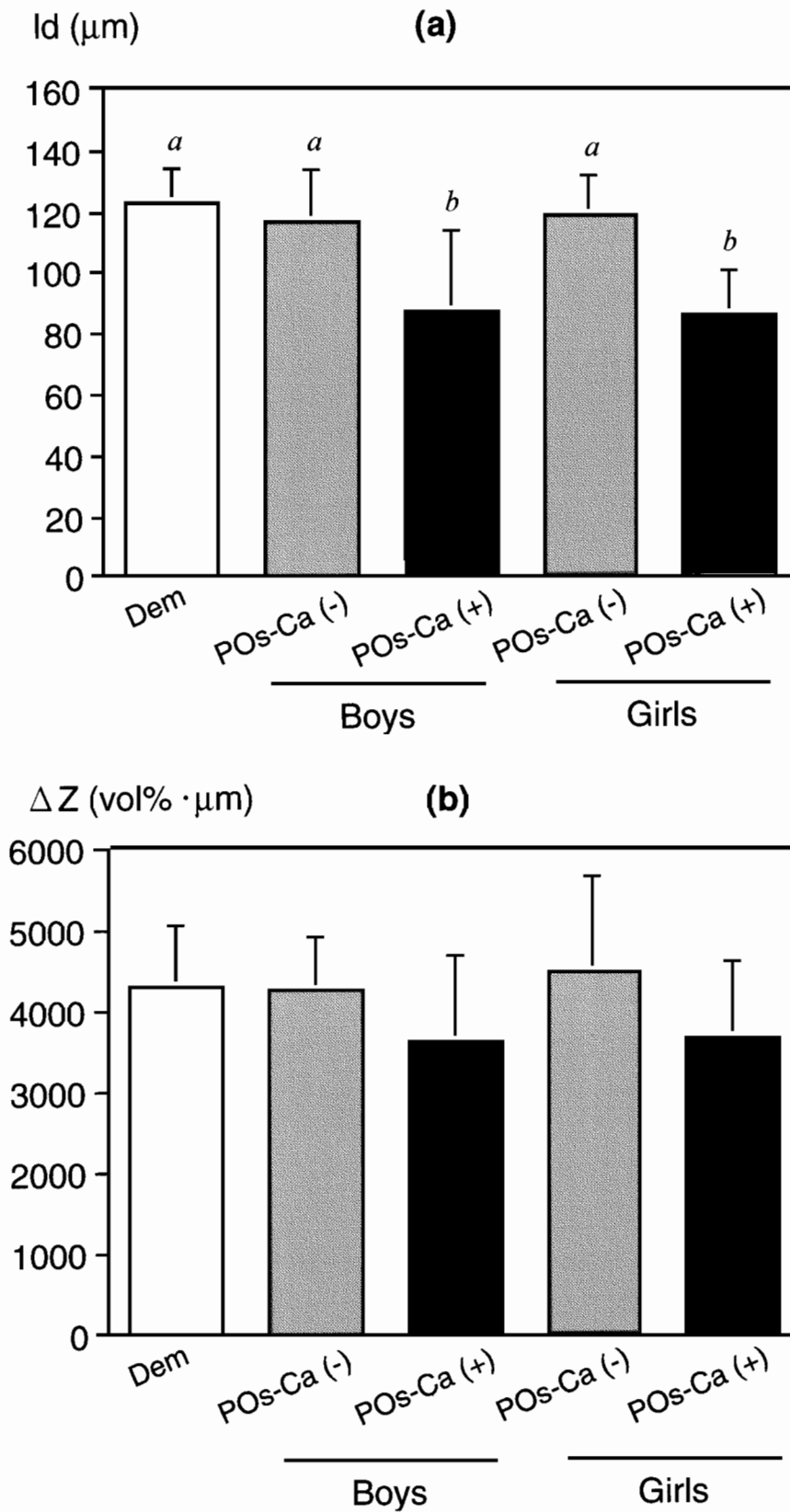


Fig. 2. Comparison of Id (a) and ΔZ (b) Values Divided into Boys (n = 5) and Girls (n = 8) in both POs-Ca (+) and POs-Ca (-) Gum.

Values are means $\pm$ SD. Dem, demineralization. ^{a,b}Values not sharing a common superscript letters are significantly different ($p < 0.05$).

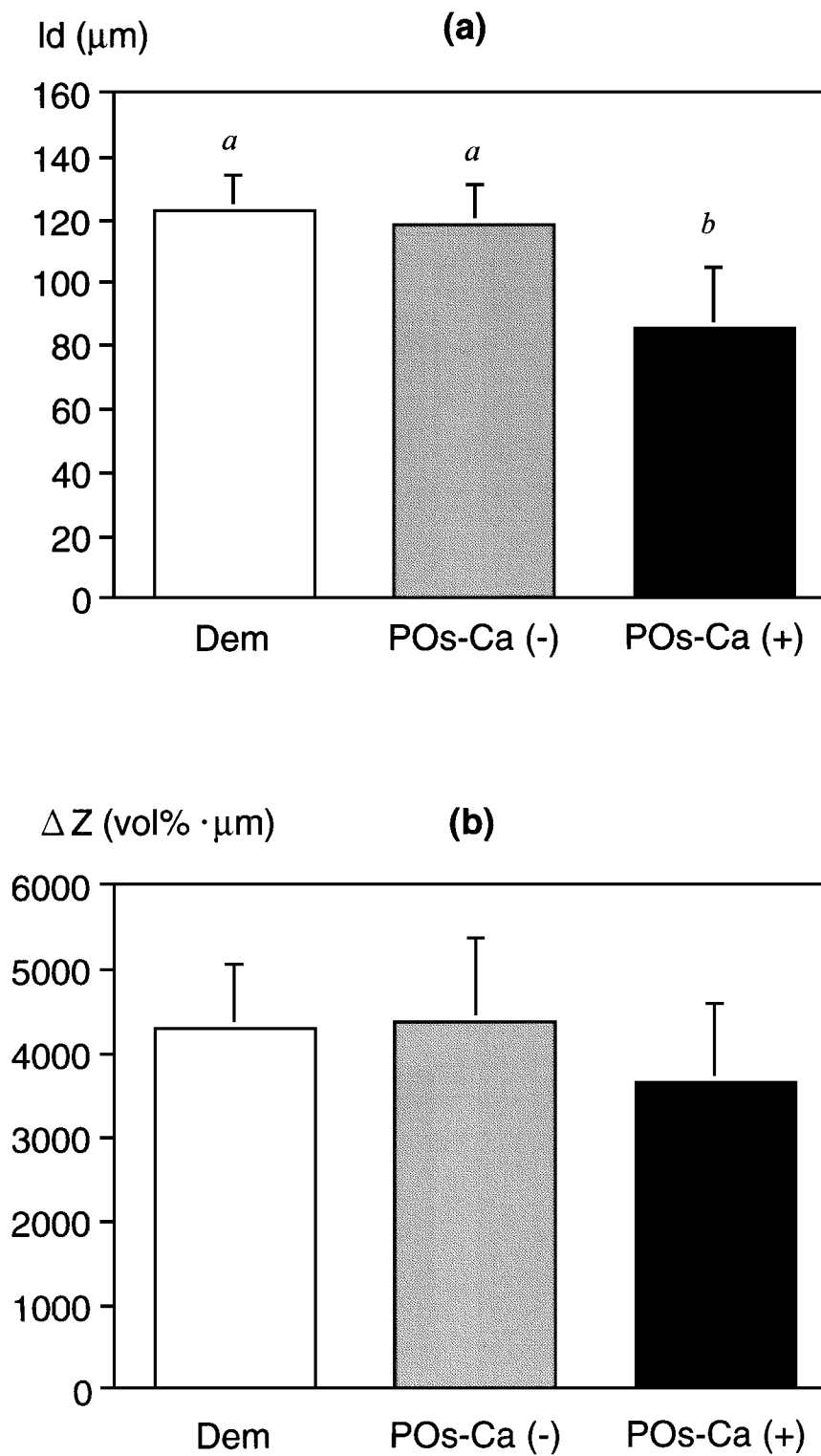


Fig. 3. Comparison of Id (a) and ΔZ (b) Values by the Types of Gum.

Values are means $\pm$ SD. Dem, demineralization. ^{*a,b*}Values not sharing a common superscript letters are significantly different ($p < 0.01$).

4. Discussion

In this Chapter, the author demonstrated that the collected whole saliva of children volunteers (7-15 yr) when chewing one tablet of sugar-free gum containing 2.5% POs-Ca (POs-Ca (+) gum) for 10 min significantly enhanced the remineralization of artificial early lesions in enamel by the *in vitro* human saliva immersing (HSI) test.

Inaba *et al.* (2002a, 2002b, 2002c, 2003) and Kamasaka *et al.* (2002) have shown the remineralization effect of POs-Ca (+) gum. However, all the study was carried out by adult volunteers and there was no data of the remineralization effect of POs-Ca (+) gum in children. Therefore, the present HSI-test with children volunteers was carried out. Although it was desirable that the experiment would be carried out by the intraoral study equipped with the oral device, in this case, the intraoral study was substantially difficult since the volunteers were schoolchildren. Thus, the remineralization effect of POs-Ca (+) gum in children was evaluated by the HSI-test. Collection of stimulated whole saliva from the volunteers and immersion of demineralized bovine enamel slabs into the saliva were carried out at each home under the supervision of their parents. The parents were fully explained in advance the procedures of the study and agreed with participation of their children to the study. Nevertheless, the number of enamel slabs subjected to the analysis of mineral distribution (total n = 13) decreased from the start of this study due to incomplete recovery of enamel slabs from the volunteers. Despite the task of HSI-test is fewer for the volunteers than the intraoral study, 9 volunteers retired during the test.

As to the mineral recovery, it was indicated that the mineral recovery (remineralization) seemed to occur mainly the lesion bottom because reduction of Id was more remarkable than that of ΔZ in the POs-Ca (+) gum group (Fig. 2 and 3).

From the results of the HSI-test with adults (Kamasaka *et al.*, 2002), it was demonstrated that the enhanced remineralization in POs-Ca (+) gum group was considered to be the results of increased salivary calcium concentration and of increased molar ratio of salivary Ca/P nearly up to that of hydroxyapatite (1.67) even at elevated salivary pH, because the salivary phosphate concentration in both POs-Ca (-) and POs-Ca (+) gum groups were equivalent and enough for the remineralization. The salivary calcium and phosphate concentrations in the children were almost consistent with the study of Kavanagh and Svehla (1998).

In this study, the significant increase in salivary calcium when chewing POs-Ca (+) gum was observed in boys and girls, as shown in Table 2. This result was consistent with the result in the HSI-test with adults (Table 3). Therefore, the enhanced remineralization in children's saliva stimulated by POs-Ca (+) gum was also caused by the elevated salivary calcium and Ca/P ratio. Although there was a difference in the number of gum chewed between this study with children (one tablet) and with adults (two tablets), the ratio of salivary calcium with mean values in POs-Ca (+) gum group/POs-Ca (-) gum group in the children and in the adults were almost equal, as shown in Table 3; $2.98 (= 4.69/1.57)$ and $2.85 (= 5.75/2.02)$, respectively. Thus, it was suggested that the increase in salivary calcium and Ca/P ratio by chewing one tablet of POs-Ca (+) gum was enough to remineralize the enamel lesion in children due to the ability of POs-Ca to maintain the calcium solubility at neutral pH (Kamasaka *et al.*, 1995 and Chapter II, section 2, Table 4).

The stimulated salivary pH in children were relatively higher than those in adults (Kamasaka *et al.*, 2002), and the salivary pH in children were almost consistent with the study of O'Sullivan *et al.* (2000). The pH around 7.5 is suitable for remineralization, but is not preferable for calcium and phosphate to be ionized (Kamasaka *et al.*, 1995).

Table 3. Comparison of Properties of Saliva Collected When Chewing Gum for 10 min between Children and Adults

Types of gum		Children (n = 22)	Adults (n = 6)**
No. of tablet chewed		1	2
Volume (mL)	POs-Ca (+)	11.09±5.13	20.17±4.98
	POs-Ca (-)	10.68±4.46	19.46±5.10
pH	POs-Ca (+)	7.92±0.28	-
	POs-Ca (-)	7.82±0.20	-
Ca (mM)	POs-Ca (+)	4.69±1.14*	5.75±0.91*
	POs-Ca (-)	1.57±0.30	2.02±0.17
P (mM)	POs-Ca (+)	5.63±0.94	5.31±0.83
	POs-Ca (-)	5.38±1.30	6.69±1.21
Ca/P ratio	POs-Ca (+)	0.85±0.22*	1.09±0.18*
	POs-Ca (-)	0.30±0.06	0.31±0.05

Values are means ± SD. *POs-Ca (+) vs. POs-Ca (-) ($p < 0.01$).

**Data of adults were referred from Kamasaka *et al.* (2002).

Inaba *et al.* (2002a) demonstrated the possible main mechanisms for enhanced remineralization in POs-Ca (+) gum, as shown in Fig. 4. Mastication of POs-Ca (+) gum stimulates strongly salivary flow, and increases calcium concentration in saliva. Simultaneously, salivary pH rises from about 7.0 to 7.5 (Kamasaka *et al.*, 2002). The rise in salivary pH is suitable for remineralization in general, but is not advantageous to maintain the solubility of calcium by rapid formation of insoluble calcium-phosphate complex in such alkaline pH. Therefore, in the presence of POs-Ca, the formation of calcium-phosphate complex is inhibited by POs-Ca and soluble calcium in saliva increases efficiently. Thus, the increase in the salivary Ca/P ratio results in remineralization of enamel lesion.

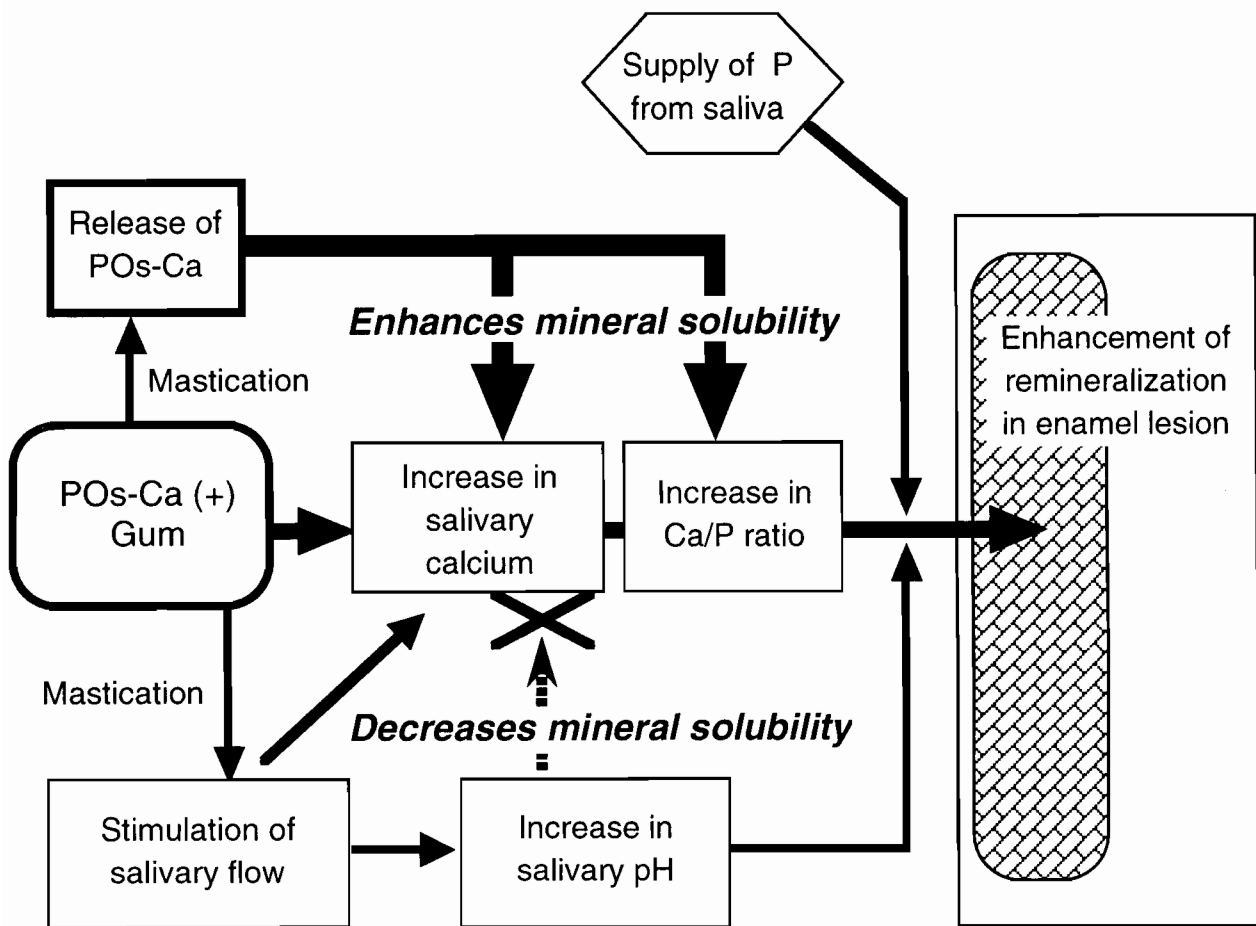


Fig. 4. Diagram Showing the Possible Mechanisms of Intraoral Enhancement of Enamel Remineralization by POs-Ca Supplied by Chewing Gum.

This diagram was referred from Inaba *et al.* (2002a) with slight modifications.

It was interesting that the I_d and ΔZ values in POs-Ca (-) gum group result in no significant difference compared with just after the initial demineralization. This result suggested that xylitol supplied by chewing gum may not directly contribute to remineralization of enamel lesion. In general, chewing gums containing xylitol was introduced as having beneficial effects in promoting enamel remineralization (Manning *et al.*, 1992 and Edgar, 1998). On the contrary, Wennerholm *et al.* (1994) and Iijima *et al.* (1999) demonstrated the negative intraoral effects of xylitol in enhancing enamel remineralization. Therefore, the remineralization effects of xylitol on intraoral enamel lesion are controversial at present.

In addition, as to the digestibility of POs-Ca, Takii *et al.* (2003) demonstrated that the gastrointestinal condition of volunteers ($n = 35$, 33 years old of mean age) was not influenced even after a one-shot oral administration of 2.6 g of POs-Ca corresponding to the amount in 70 tablets of POs-Ca (+) gum. From this result and the digestibility of POs-Ca in rats as described in Chapter II, section 1, the author considers that the amount of POs-Ca from POs-Ca (+) gum does not cause acute diarrhea in an ordinary use of POs-Ca (+) gum.

In conclusion, the author consider that the habitual use of POs-Ca (+) gum will be beneficial for not only caries prevention but also enhancement of remineralization in children who have more opportunities for intake of fermentable saccharides such as sucrose and glucose from daily meals or snacks, and in pregnant women and new mothers.

5. Summary

The effect of whole saliva of child volunteers stimulated by chewing the sugar-free chewing gum containing 2.5% of phosphoryl oligosaccharides of calcium (POs-Ca) on remineralization of enamel *in vitro* was investigated. The stimulated whole saliva was collected from 22 children volunteers (9 boys and 13 girls) by chewing one tablet of sugar-free gum with POs-Ca (POs-Ca (+) gum) or without POs-Ca (POs-Ca (-) gum) for 10 min. Demineralized bovine enamel slabs were immersed in the collected saliva for 40 min at 37°C. This treatment was repeated for successive 4 days. The enamel slabs after 4-d treatments were microradiographed, and the mineral distribution was analyzed. The salivary calcium concentration of POs-Ca (+) gum group was significantly higher than that of POs-Ca (-) gum group both in boys and girls. The phosphate concentrations of both groups were not significantly different. The lesion depth (ld) value was significantly lower for the POs-Ca (+) gum group than that of the POs-Ca (-) gum group both in boys and girls. The ld values were $86 \pm 27 \mu\text{m}$ (means $\pm$ SD, boys in the POs-Ca (+) gum group), $85 \pm 15 \mu\text{m}$ (girls in the POs-Ca (+) gum group), $117 \pm 16 \mu\text{m}$ (boys in the POs-Ca (-) gum group) and $118 \pm 12 \mu\text{m}$ (girls in the POs-Ca (-) gum group). These results indicate that daily application of sugar-free chewing gum containing 2.5% of POs-Ca enhances remineralization of enamel lesion, especially for children who have higher risk of demineralization than adults and is useful for caries prevention.

CONCLUSION

This thesis deals with a novel property of a fungal acid phosphatase for phosphate ester groups of phosphoryl oligosaccharides (POs), with bioavailability of phosphoryl oligosaccharides of calcium (POs-Ca) as a calcium material for foods and with application of POs-Ca to chewing gum as a remineralization enhancer. The results and findings obtained can be concludes as follow.

1. Characterization of acid phosphatase to understand the function of POs

In Chapter I, the author screened for the specifically dephosphorylating enzyme of C-3 or C-6 phosphate groups of POs in microorganism, and isolated a fungus. This fungus produced two types of intracellular acid phosphatase (ACPase): ACPase I and II. Of the two phosphatases, ACPase II was a minor component, but only this enzyme preferentially dephosphorylated C-6 rather than C-3 phosphate group of POs. ACPase II was purified from the cell-free extract of *Aspergillus niger* KU-8. The optimum pH and temperature of the enzyme were 2.0 and 60 °C, respectively.

From the result of the course of reaction of ACPase II on POs, it is indicated that the dephosphorylation rate of C-6 phosphate groups of POs by ACPase II is dependent on the degrees of polymerization (DP) of 6-phosphoryl oligosaccharides, namely, the lower DP of 6-phosphoryl oligosaccharides, the faster ACPase II dephosphorylates phosphate group of 6-phosphoryl oligosaccharides.

In addition, the author also described the kinetic parameters of ACPase II toward C-3 and C-6 phosphate groups of POs. The author prepared 3²-phosphoryl maltotriose and 6²-phosphoryl maltotriose from POs. They

have the same DP and only differ in the binding position of the phosphate group. The K_m for both substrates were similar. However, the k_{cat} value for the 6²-phosphoryl maltotriose was about 3-fold of that for the 3²-phosphoryl maltotriose. These results indicated that the difference of ACPase II specificity toward C-3 or C-6 phosphate groups of phosphoryl oligosaccharides was due to the difference of k_{cat} .

2. Application of POs-Ca as soluble calcium material

In Chapter II, the author described bioavailability of POs-Ca. The solubility of POs-Ca as calcium was compared with calcium compounds of soluble (calcium chloride calcium lactate) and insoluble (calcium carbonate and calcium phosphate) forms. The solubility of POs-Ca was as high as that of calcium chloride and about 3-fold higher than that of calcium lactate. POs-Ca was not hydrolyzed by artificial gastric acid. The overall rate of plasma glucose response after oral administration of POs-Ca to rats was similar to that in the case of administration of control saccharides, namely, glucose and high-maltotetraose syrup (TETRUP-H). In the case of POs-Ca administration, plasma calcium concentration showed slight but significant increase during 0.25-0.5 h concurrent with the increase of plasma glucose. On the other hand, plasma phosphate concentration decreased and then increased gradually from 2 h after the administration. From these results, it was suggested that the POs-Ca orally administered would be hydrolyzed into glucose, calcium, and phosphate and then absorbed completely in the small intestine.

In addition, the author also described the *in situ* absorbability of POs-Ca as a calcium material. Calcium compounds of soluble (calcium chloride calcium lactate) and insoluble (calcium carbonate and calcium phosphate) forms were used for comparison. The solubility of POs-Ca

was as high as that of calcium chloride and about 3-fold higher than that of calcium lactate. An *in situ* experiment showed that the intestinal calcium absorption rate of POs-Ca was almost equal to that of the soluble calcium compounds, but the absorption rate was significantly higher than those of the insoluble calcium groups. Furthermore, the total absorption rate of a 1:1 mixture of the calcium from POs-Ca and a whey mineral complex (WMC) was significantly higher than that of WMC alone.

Growth of rats fed the diet containing 10% POs-Ca for 15 days was not different from those of rats fed control diet, and no diarrhea was observed in the POs-Ca group. Apparent calcium absorption and retention rates between the groups were not significantly different. These results indicated that POs-Ca would be a useful soluble calcium material with relatively high absorption in the intestinal tract.

3. Application of POs-Ca as remineralization enhancer

In Chapter III, the author described remineralization effect of sugar-free chewing gum containing 2.5% POs-Ca child volunteers on enamel lesion *in vitro*. The stimulated whole saliva was collected from 22 children volunteers (9 boys and 13 girls) by chewing one tablet of sugar-free gum with POs-Ca (POs-Ca (+) gum) or without POs-Ca (POs-Ca (-) gum) for 10 min. Demineralized bovine enamel slabs were immersed in the collected saliva for 40 min at 37°C. This treatment was repeated for successive 4 days. The salivary calcium concentration of POs-Ca (+) gum group was significantly higher than that of POs-Ca (-) gum group in both boys and girls. The phosphate concentrations of both groups were not significantly different. The lesion depth value of enamel slabs was significantly lower for the POs-Ca (+) gum group than that of the POs-Ca (-) gum group in both boys and girls. These results indicated that daily application of sugar-free

chewing gum containing 2.5% POs-Ca significantly enhanced remineralization of enamel lesion not only for adults but also for children. Children generally have more opportunities for intake of fermentable saccharides such as sucrose and glucose from daily meals or snacks.

4. Further works

The author and co-workers recently succeeded in finding the POs in the waste water generated in the saccharifying process of potato starch, so that POs could be obtained on an industrial scale. However, whether the difference of the binding position of phosphate groups affects the function of POs is not clear so far. The characterization of ACPase II and 3-phosphoryl oligosaccharides prepared from POs by using ACPase II may help to understand the characteristics of other acid and alkaline phosphatases and phosphate groups existing in various starches.

In diabetic complications, C-3 phosphorylated saccharides, sorbitol 3-phosphate and fructose 3-phosphate, have been detected by ^{31}P -NMR in diabetic in human erythrocyte and in mammalian lens as diabetes-associated metabolites (Szwergold *et al.*, 1989, 1990, Petersen *et al.*, 1990, 1992). These C-3 phosphorylated saccharides are produced by an unknown specific 3-phosphokinase that phosphorylates saccharides at the C-3 hydroxyl position (Lal *et al.*, 1993), may be relevant to the non-enzymatic glycosylations of protein. These advanced glycation endproducts accumulate in lens and cause diabetic cataract, the opacification of lens (Szwergold *et al.*, 1989, 1990). In addition, glucose 3-phosphate is thought to be a potential intermediate in the biosynthesis of sorbitol 3-phosphate and fructose 3-phosphate (Canales *et al.*, 1994). In hyperglycemia, glucose excessively incorporated into cells is converted by

polyol pathway as follows; glucose→sorbitol→fructose (Kador, 1988). Fructose was responsible for the non-enzymatic glycation agent (Suarez, *et al.*, 1988), but fructose 3-phosphate is more potent glycation agent (Szwergold *et al.*, 1990).

Thus, 3-phosphoryl oligosaccharides or its single component are thought to be very useful for better understanding of the biological and physiological roles of C-3 phosphorylated saccharides in diabetic complications, which are not yet commercially available.

With respect to calcium absorption *in vivo*, it has been reported that increase of calcium absorption in the intestine in rats by using several indigestible oligosaccharides such as lactose (Armbrecht and Wasserman, 1976), fructooligosaccharides (Ohta *et al.*, 1993), galactooligosaccharides (Chonan *et al.*, 1995), or a sugar alcohol of maltose, namely, maltitol (Goda *et al.*, 1992). The effects of those indigestible oligosaccharides including sugar alcohols on calcium absorption in the intestine may involve the production of short-chain fatty acids (SCFAs), namely, acetic acid, propionic acid and butyric acid, by microbial fermentation of indigestible oligosaccharides in the large intestine. The SCFAs stimulate the proliferation of epithelial cells in the intestine (Sakata, 1987) and reduce luminal pH (Demigne *et al.*, 1989, Ohta *et al.*, 1994, Younes *et al.*, 1996). On the other hand, Goda *et al.* (1993) demonstrated that maltitol enhanced the rate of *in vitro* transepithelial calcium transport in the ileum (lower part of small intestine) by modulating the passive diffusion of calcium using the rat everted intestinal sac method. This finding suggests that indigestible oligosaccharides could enhance calcium absorption by a mechanism independent of fermentation in the large intestine.

In addition, as mentioned in Chapter II, section 2, phosphorylated guar gum hydrolysate (P-GGH) inhibited the precipitation of calcium-phosphate *in vitro* (Watanabe *et al.*, 2000). The apparent calcium absorption was significantly higher in the rats fed 5% P-GGH diet than in the rats fed 5% guar gum hydrolysate (GGH) diet, although this effect was only found in the first period (day 4 - 7) of balance study during 14 days feeding period. GGH is a highly fermentable dietary fiber with low viscosity (Takahashi *et al.*, 1994), and the feeding of GGH to rats that lowered calcium absorption by partial nephrectomy restored fully calcium absorption (Hara *et al.*, 1996).

From these results, the author considers that not only the presence and the number of phosphate group in one molecule of POs-Ca but also the indigestibility of the molecule itself are important factors that affect the calcium absorption in the intestine. From this viewpoint, the improvement of POs-Ca or development of POs-derivatives of being able to use to foods is to be progressed.

Application of POs-Ca to foods came true by development of a sugar-free chewing gum containing POs-Ca that has a high remineralization effect in early caries lesion. This chewing gum was sanctioned as a “food for specified health uses” by Ministry of Health, Labour and Welfare on 2003. With progress of preventive dentistry in recent years, it has been recognized that the dental prevention (care) is more important than operative treatment (cure) to maintain the oral function and quality of life (QOL). In addition, the application of POs-Ca means utilization of unused agricultural resources. POs-Ca is made from the by-product generating in the saccharifying process of potato starch that is manufactured from potato cultivated in Hokkaido. Potato is one of the main crops in Hokkaido and

its yield is leveling off in recent years; consequently, the author expects that the success of application of POs-Ca to chewing gum will make small but important contribution to QOL in people and agriculture in Hokkaido.

ACKNOWLEDGEMENTS

I deeply thank Dr. Takashi Kuriki, Director of Biochemical Laboratory, Ezaki Glico Co., Ltd., for his helpful advice and warm encouragement during the course of this investigation.

I greatly appreciate to Professor Takeshi Yasuda, Faculty of Agriculture, Kobe University, for valuable guidance and helpful suggestions, and great encouragement through the course of this study.

Thanks are also due to Professor Takashi Nanmori, and Professor Yukihiro Sugimoto, Faculty of Agriculture, Kobe University, for their discussion of the manuscript.

I wish to thank Professor Yukihiro Nakabou, Faculty of Human Environmental Science, Kyoto Prefectural University, Associate Professor Shigeru Saeki, and Assistant Professor Dong-Ho Kim, Department of Food and Nutrition, Graduate School of Human Life Science, Osaka City University, for their teachings, helpful suggestions, and kind assistance with the investigation of calcium absorption in rats.

I would like to thank Professor Masami Yonemitsu, Associate Professor Daisuke Inaba, and Assistant Professor Kentaro Minami, School of Dentistry, Iwate Medical University, for their teachings, and helpful suggestions with the investigation of remineralization in children.

I am grateful to Mr. Katsuhisa Ezaki, President and CEO, Mr. Katsuya Okawabata, Executive Director, and Dr. Shigetaka Okada, former Director of Biochemical Laboratory, Ezaki Glico Co., Ltd., for providing me a chance of this study.

I am also deeply indebted to Dr. Takashi Kometani, Director of Central Research Laboratory, Dr. Takahisa Nishimura, Section Manager of Biochemical Research Laboratory, Dr. Hiroshi Kamasaka, Research Manager of Biochemical Research Laboratory, Ezaki Glico Co., Ltd., and many colleagues in Biochemical Research Laboratory, Ezaki Glico Co., Ltd.

I also thank Ms. Akemi Hosoda, Mr. Masanari Ozaike, and many colleagues in Osaka City University, for their help and encouragement.

I dedicate this work to my family whose help and support over the years has made it all possible.

REFERENCES

- Arends, J., Christoffersen, J., Ruben, J., and Jongebloed, W. L., Remineralization of bovine dentine *in vitro*. *Caries Res.*, **23**, 309-314 (1989).
- Arends, J., Ruben, J., Christoffersen, J., Jongebloed, W. L., and Zuidgeest, T. G. M., Remineralization of human dentin *in vitro*. *Caries Res.*, **24**, 432-435 (1990).
- Arends, J., and ten Bosch, J. J., Demineralization and remineralization evaluation techniques. *J. Dent. Res.*, **71**, 924-928 (1992).
- Armbrecht, H. J., and Wasserman, R. H., Enhancement of Ca^{++} uptake by lactose in the rat small intestine. *J. Nutr.*, **106**, 1265-1271 (1976).
- Bajwa, W., Mayhack, B., Rudolph, H., Schweigruber, A. M., and Hinnen, A., Structural analysis of the two tandemly repeated acid phosphatase genes in yeast. *Nucleic Acids Res.*, **12**, 7721-7739 (1984).
- Barbaric, S., Kozulic, B., Ries, B., and Mildner, P., Physicochemical and kinetic parameters of acid phosphatase from *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **259**, 878-883 (1984).
- Berrocal, R., Chanton, S., Juillerat, M. A., Scherz, J. C., and Jost, R., Tryptic phosphopeptides from whole casein II. Physicochemical properties related to solubilization of calcium. *J. Dairy Res.*, **56**, 335-341 (1989).
- Blennow, A., Bya-Smith, A. M., Olsen, C. E., and Møllar, B. L., Analysis

of starch-bound glucose 3-phosphate and glucose 6-phosphate using controlled acid treatment combined with high-performance anion-exchange chromatography. *J. Chromatogr.*, **829**, 385-391 (1998).

Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248-54 (1976).

Bronner, F., Calcium absorption - A paradigm for mineral absorption. *J. Nutr.*, **128**, 917-920 (1998).

Bronner, F., and Pansu, D., Nutritional aspects of calcium absorption. *J. Nutr.*, **129**, 9-12 (1999).

Bronner, F., Pansu, D., and Stein, W. D., An analysis of intestinal calcium transport across the rat intestine. *Am. J. Physiol.*, **250**, G561-G569 (1986).

Canales, J., Buitrago, F., Faraldo, A., and Cameselle, J. C., Detection of specific glucose-3-phosphatase activity in rat liver. *FEBS Lett.*, **339**, 55-58 (1994).

Cashman, K. D., Calcium intake, calcium bioavailability and bone health. *Br. J. Nutr.*, **87**, S169-S177 (2002).

Chonan, O., and Watanuki, M., Effect of galactooligosaccharides on calcium absorption in rats. *J. Nutr. Sci. Vitaminol.*, **41**, 95-104 (1995).

Demigne, C., Levrat, M. A., and Remesy, C., Effect of feeding fermentable carbohydrates on the cecal concentrations of mineral and

- their fluxes between the cecum and blood plasma in rats. *J. Nutr.*, **119**, 1625-1630 (1989).
- Dowd, F. J.**, Saliva and dental caries. *Dent. Clin. North Am.*, **43**, 579-597 (1999).
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F.**, Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, **28**, 350-356 (1956).
- Duflos, C., Bellaton, C., Pansu, D., and Bronner, F.**, Calcium solubility, intestinal sojourn time and paracellular permeability codetermine passive calcium absorption in rats. *J. Nutr.*, **125**, 2348-2355 (1995).
- Edgar, W. M., Higham, S. M., and Manning, R. H.**, Saliva stimulation and caries prevention. *Adv. Dent. Res.*, **8**, 239-245 (1994).
- Edgar, W. M.**, Sugar substitutes, chewing gum and dental caries - A review. *Br. Dent. J.*, **184**, 29-32 (1998).
- Goda, T., Yamada, M., Takase, S., and Hosoya, N.**, Effect of maltitol intake on intestinal calcium absorption in rats. *J. Nutr. Sci. Vitaminol.*, **38**, 277-286 (1992).
- Goda, T., Takase, S., and Hosoya, N.**, Maltitol-induced increase of transepithelial transport in rat small intestine. *J. Nutr. Sci. Vitaminol.*, **39**, 589-595 (1993).
- Goda, T., and Oku, T.**, in "Digestion and Absorption", eds. Muto, Y., Daiichi Shuppan, Tokyo, pp235-295 (2002).

- Gracza, R.,** Minor constituents of starch, in “Starch chemistry and technology I”, eds. Whistler, R. J., and Paschall, E. F., Academic Press, New York, pp105- 131 (1965).
- Gunshin, H., Naito, H., Noguchi, T., Todoroki, H., and Akamatsu, T.,** Effect of Ca level on ⁶⁵Zn absorption at the ligated duodenal loop of rat. *Ann. Physiol. Anthropol. (in Japanese)*, **8**, 227-231 (1989).
- Haas, H., Redl, B., Leitner, E., and Stoffler, G.,** *Penicillium chrysogenum* extracellular acid phosphatase: purification and biochemical characterization. *Biochim. Biophys. Acta*, **1074**, 392-397 (1991).
- Hansen, M., Sandstrom, B., Jensen, M., and Sorensen, S. S.,** Effects of casein phosphopeptides on zinc and calcium absorption from bread meals. *Trace Elem. Med. Biol.*, **11**, 143-149 (1997).
- Hara, H., Nagata, M., Ohta, A., and Kasai, T.,** Increases in calcium absorption with ingestion of soluble dietary fibre, guar-gum hydrolysate, depend on the calcium in partially nephrectomized and normal rats. *Br. J. Nutr.*, **76**, 773-784 (1996).
- Heaney, R. P., Recker, R. R., and Weaver, C. M.,** Absorbability of calcium sources: the limited role of solubility. *Calcif. Tissue Int.*, **46**, 300-304 (1990).
- Hicks, J., Garcia-Godoy, F., and Flaitz, C.,** Biological factors in dental caries: role of saliva and dental plaque in the dynamic process of demineralization and remineralization (part 1). *J. Clin. Pediatr. Dent.*, **28**, 47-52 (2003).

- Hicks, J., Garcia-Godoy, F., and Flaitz, C.,** Biological factors in dental caries enamel structure and the dynamic process of demineralization and remineralization (part 2). *J. Clin. Pediatr. Dent.*, **28**, 119-124 (2004).
- Hizukuri, S., Tabata, S., and Nikuni, Z.,** Studies on starch phosphate part 1. Estimation of glucose-6-phosphate residues in starch and the presence of other bound phosphate(s). *Stärke*, **22**, 338-343 (1970).
- Hollander, V. P.,** in “The enzymes”, eds. Boyer, P. D., Academic Press, New York, pp57-77 (1971).
- Iijima, Y., and Takagi, O.,** The evaluation of *in situ* remineralization potential of sugar substitutes. *J. Dent. Hlth. (in Japanese)*, **49**, 803-808 (1999).
- Inaba, D., Takagi, O., and Arends, J.,** Computer-assisted videodensitometric method to visualize mineral distributions in *in vitro* and *in vivo* formed root caries lesions. *Eur. J. Oral Sci.*, **105**, 74-84 (1997a).
- Inaba, D., Tanaka, R., Takagi, O., Yonemitsu, M., and Arends, J.,** Computerized measurements of microradiographic mineral parameters of de- and remineralized dental hard tissues. *J. Dent. Hlth.*, **47**, 67-74 (1997b).
- Inaba, D., Kamasaka, H., Minami, K., Nishimura, T., Kuriki, T., Imai, S., and Yonemitsu, M.,** Remineralization of enamel by phosphoryl-oligosaccharides (POs) supplied by chewing gum; Part II. Intraoral evaluation. *J. Dent. Hlth.*, **52**, 112-118 (2002a).

Inaba, D., Minami, K., Kamasaka, H., and Yonemitsu, M., Effect of phosphoryl-oligosaccharides (POs) on remineralization of enamel lesions *in vitro*. *Dent. J. Iwate Med. Univ.*, **27**, 197-202 (2002b).

Inaba, D., Minami, K., Kamasaka, H., and Yonemitsu, M., Remineralization of enamel and dentin by a chewing gum containing phosphoryl oligosaccharide calcium (POs-Ca) *in situ*. *Dent. J. Iwate Med. Univ.*, **27**, 203-209 (2002c).

Inaba, D., Minami, K., Kamasaka, H., Kuriki, T., Imai, S., and Yonemitsu, M., Intraoral effects of phosphoryl oligosaccharide calcium on remineralization of enamel lesions. *J. Dent. Hlth.*, **53**, 8-12 (2003).

Institute of Medicine., in “Dietary Reference Intakes: calcium, magnesium, phosphorus, vitamin D, and fluoride.”, Washington, DC: Food and Nutrition Board, National Academy Press. (1997).

Irving, G. C. J., and Cosgrove, D. J., Inositol phosphate phosphatases of microbiological origin. Some properties of the partially purified phosphatases of *Aspergillus ficuum* NRRL 3135. *Aust. J. Biol. Sci.*, **27**, 361-368 (1974).

Itaya, K., and Ui, M., A new micromethod for the colorimetric determination of inorganic phosphate. *Clin. Chim. Acta*, **14**, 361-366 (1966).

Itthagarun, A., Chewing gum and saliva in oral health. *J. Clin. Dent.*, **8**, 159-162 (1997).

Kador, P. F., The role of aldose reductase in the development of diabetic

complications. *Med. Res. Rev.*, **8**, 325-352 (1988).

Kamasaka, H., Uchida, M., Kusaka, K., Yoshikawa, K., Yamamoto, K., Okada, S., and Ichikawa, T., Inhibitory effect of phosphorylated oligosaccharides prepared from potato starch on the formation of calcium phosphate. *Biosci. Biotechnol. Biochem.*, **59**, 1412-1416 (1995).

Kamasaka, H., To-o, K., Kusaka, K., Kuriki, T., Kometani, T., Hayashi, H., and Okada, S., The structures of phosphoryl oligosaccharides prepared from potato starch. *Biosci. Biotechnol. Biochem.*, **61**, 238-244 (1997a).

Kamasaka, H., To-o, K., Uchida, M., Kusaka, K., Kuriki, T., Kometani, T., Hayashi, H., Okada, S., and Ichikawa, T., Studies of phosphoryl oligosaccharides prepared from potato starch. *Oyo Toshitsu Kagaku (J. Appl. Glycosci.)*, **44**, 253-261 (1997b).

Kamasaka, H., To-o, K., Kusaka, K., Kuriki, T., Kometani, T., Hayashi, H., and Okada, S., Action pattern of neopullulanase on phosphoryl oligosaccharides prepared from potato starch. *Oyo Toshitsu Kagaku (J. Appl. Glycosci.)*, **44**, 275-283 (1997c).

Kamasaka, H., To-o, K., Kusaka, K., Kuriki, T., Kometani, T., and Okada, S., A way of enhancing the inhibitory effect of phosphoryl oligosaccharides on the formation of a calcium phosphate precipitate using the coupling reaction of cyclodextrin glucanotransferase. *Oyo Toshitsu Kagaku (J. Appl. Glycosci.)*, **44**, 285-293 (1997d).

Kamasaka, H., Inaba, D., Minami, K., Nishimura, T., Kuriki, T., Imai, S., and Yonemitsu, M., Remineralization of enamel by

phosphoryl-oligosaccharides (POs) supplied by chewing gum; Part I. Salivary assessment in vitro. *J. Dent. Hlth.*, **52**, 105-111 (2002).

Kaneko, T., Yokoyama, A., and Suzuki, M., Digestibility of isomaltooligosaccharides by rats and effects on serum lipids. *Nippon Nogeikagaku Kaishi (in Japanese)*, **66**, 1211-1220 (1992).

Kashket, S., Historical review of remineralization research. *J. Clin. Dent.*, **10**, 56-64 (1999).

Kavangh, D. A., and Svehla, G., Variation of salivary calcium, phosphate and buffering capacity in adolescents. *Archs. Oral Biol.*, **43**, 1023-1027 (1998).

Kilsheimer, G. S., and Axlerod, B., Inhibition of prostatic acid phosphatase by alpha-hydroxycarboxylic acids. *J. Biol. Chem.*, **227**, 879-890 (1957).

Komano, T., Formation of multiple forms of acid and alkaline phosphatases in relation to the culture age of *Aspergillus niger*. *Plant Cell Physiol.*, **16**, 643-635 (1975).

Kostrewa, D., Wyss, M., D'Arcy, A., and van Loon, A. P. G. M., Crystal structure of *Aspergillus niger* pH 2.5 acid phosphatase at 2.4 Å resolution. *J. Mol. Biol.*, **288**, 965-974 (1999).

Kuriki, T., Okada, S., and Imanaka, T., New type of pullulanase from *Bacillus stearothermophilus* and molecular cloning and expression of the gene in *Bacillus subtilis*. *J. Bacteriol.*, **170**, 1554-1559 (1988).

Kuriki, T., and Okada, S., in "Enzyme chemistry and molecular biology of

amylases and related enzymes”, eds. The Amylase Research Society of Japan, CRC Press, Florida, pp28-32 (1995).

Kuriki, T., Kaneko, H., Yanase, M., Takata, H., Shimada, J., Handa, S., Takada, T., Umeyama, H., and Okada, S., Controlling substrate preference and transglycosylation activity of neopullulanase by manipulating steric constraint and hydrophobicity in active center. *J. Biol. Chem.*, **271**, 17321-17329 (1996).

Lacour, B., Tardivel, S., and Druke, T., Stimulation by citric acid of calcium and phosphorus bioavailability in rats fed a calcium-rich diet. *Miner. Electrolyte Metab.*, **23**, 79-87 (1997).

Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685 (1970).

Lal, S., Szwergold, B. S., Kappler, F., and Brown, T. R., Detection of fructose-3-phosphokinase activity in intact mammalian lens by ³¹P NMR spectroscopy. *J. Biol. Chem.*, **268**, 7763-7767 (1993).

Lee, S. Y., Park, G., and Naito, H., Supplemental effect of casein phosphopeptides (CPP) on the calcium valance of rowing rats. *Nippon Eiyo Shokuryo Gakkaishi (in Japanese)*, **45**, 433-439 (1992).

Levenson, D. I., and Bockman, R. S., A review of calcium preparations. *Nutr. Rev.*, **52**, 221-232 (1994).

Luchter-Wasylewska, E., Continuous assay for acid phosphatase using phenyl phosphate. *Anal. Biochem.*, **241**, 167-172 (1996).

Manning, R. H., Edgar, W. M., and Agalamanyi, E. A., Effects of chewing gums sweetened with sorbitol or a sorbitol/xylitol mixture on the remineralization of human enamel lesions *in situ*. *Caries Res.*, **26**, 104-109 (1992).

Manning, R. H., and Edgar, W. M., pH Changes in plaque after eating snacks and meals, and their modification by chewing sugared- or sugar-free gum. *Br. Dent. J.*, **174**, 241-244 (1993).

McTigue, J. J., and Van Etten, R. L., An essential arginine residue in human prostatic acid phosphatase. *Biochim. Biophys. Acta*, **523**, 4422-429 (1978).

Naito, H., The mechanism of enhancement in intestinal calcium absorption with phosphopeptides derived during casein digestion. *Nippon Eiyo Shokuryo Gakkaishi (in Japanese)*, **39**, 433-439 (1986).

National Research Council., Recommended dietary Allowances: 10th edition. Report of the subcommittee on the tenth edition of the RDA Food and Nutrition Board and the Commission on Life Sciences, Washington DC: National Academy Press. (1989).

Nelson, N., A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.*, **153**, 375-380 (1944).

Nishijima, K., and Okano, T., Intestinal absorption of calcium ascorbate in rats. *J. Bone Miner. Metab.*, **17**, 30-36 (1999).

Nielsen, T. H., Wischman, K., Enevolsden, K., and Moller, B. L., Starch phosphorylation in potato tubers proceeds concurrently with *de novo*

- biosynthesis of starch. *Plant Physiol.*, **105**, 111-117 (1994).
- Nordin, B. E.**, Calcium in health and disease. *Food Nutr. Agric.*, **20**, 13-26 (1997).
- Ohta, A., Osakabe, N., Yamada, K., Saito, Y., and Hidaka, H.**, Effect of fructooligosaccharides and other saccharides on Ca, Mg and P absorption in rats. *J. Jpn. Soc. Nutr. Food Sci.*, **46**, 123-129 (1993).
- Ohta, A., Baba, S., Takizawa, T., and Adachi, T.**, Effects of fructooligosaccharides on the absorption of magnesium in the magnesium-deficient rat model. *J. Nutr. Sci. Vitaminol.*, **40**, 171-180 (1994).
- Olczak, M., Watorek, W., and Morawiecka, B.**, Purification and characterization of acid phosphatase from yellow lupin (*Lupinus luteus*) seeds. *Biochim. Biophys. Acta*, **1341**, 14-25 (1997).
- O'Sullivan, E. A., and Curzon, M. E. J.**, Salivary factors affecting dental erosion in children. *Caries Res.*, **34**, 82-87 (2000).
- Pak, C. Y. C., and Avioli, L. V.**, Factors affecting absorbability of calcium from calcium salts and food. *Calcif. Tissue Int.*, **43**, 55-60 (1988).
- Pansu, D., Bellaton, C., Roche, C., and Bronner, F.**, Theophylline inhibits transcellular Ca transport in intestine and Ca-binding by CaBP. *Am. J. Physiol.*, **257**, G935-G943 (1989).
- Pansu, D., Duflos, C., Bellaton, C., and Bronner, F.**, Solubility and intestinal transit time limit calcium absorption in rats. *J. Nutr.*, **123**,

1396-1404 (1993).

Petersen, A., Szwergold, B. S., Kappler, F., Weingarten, M., and Brown, T. R., Identification of sorbitol 3-phosphate and fructose 3-phosphate in normal and diabetic human erythrocytes. *J. Biol. Chem.*, **265**, 17424-17427 (1990).

Petersen, A., Kappler, F., Szwergold, B. S., and Brown, T. R., Fructose metabolism in human erythrocyte. Phosphorylation to fructose 3-phosphate. *Biochem. J.*, **284**, 363-366 (1992).

Recker, R. R., Calcium and achlorhydria. *N. Engl. J. Med.*, **313**, 70-73 (1985).

Reeves, P. G., Nielsen, F. H., and Fahey, Jr. G. C., AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformation of the AIN-76A diet. *J. Nutr.*, **123**, 1339-1351 (1993).

Saito, Y., Lee, Y. S., and Kimura, S., Minimum effective dose of casein phosphopeptides (CPP) for enhancement of calcium absorption in growing rats. *Internat. J. Vitam. Nutr. Res.*, **68**, 335-340 (1998).

Sakata, T., Stimulatory effect of short-chain fatty acids on epithelial cell proliferation in the rat intestine: a possible explanation for trophic effects of fermentable fibre, gut microbes and mucosal trophic factors. *Br. J. Nutr.*, **58**, 95-103 (1987).

Sato, R., Noguchi, T., and Naito, H., Casein phosphopeptide (CPP) enhances calcium absorption from the ligated segment of rat small

intestine. *J. Nutr. Sci. Vitaminol.*, **32**, 67-76 (1986).

Schachter, D., Doedle, E. B., and Schenker, H., Active transport of calcium by the small intestine of the rat. *Am. J. Phys.*, **198**, 263-268 (1960).

Schoch, T. J., Fractionation of starch by selective precipitation with butanol. *J. Am. Chem. Soc.*, **64**, 2957-2961 (1942).

Shimada, Y., Shinmyo, A., and Enatsu, T., Characterization and localization of acid phosphatases produced by *Aspergillus niger*. *J. Ferment. Technol.*, **52**, 369-377 (1974).

Shimada, Y., Shinmyo, A., and Enatsu, T., Purification and properties of one component of acid phosphatase produced by *Aspergillus niger*. *Biochim. Biophys. Acta*, **480**, 412-427 (1977).

Steinberg, S., A paradigm shift in the treatment of caries. *Gen. Dent.*, **50**, 333-338 (2002).

Suarez, G., Rajaram, R., Bhuyan, K. C., Oronsky, A. L., and Goidl, J. A., Administration of an aldose reductase inhibitor induces a decrease of collagen fluorescence in diabetic rats. *J. Clin. Invest.*, **82**, 624-627 (1988).

Suzuki, A., Shibnuma, K., Takeda, Y., Abe, J., and Hizukuri, S., Structures and pasting properties of potato starches from Jaga Kids Purple '90 and Red '90. *Oyo Toshitsu Kagaku (J. Appl. Glycosci.)*, **41**, 425-432 (1994).

- Szwergold, B. S., Kappler, F., Brown, T. R., Pfeffer, P., and Osman, S. F.,** Identification of D-sorbitol 3-phosphate in the normal and diabetic mammalian lens. *J. Biol. Chem.*, **264**, 9278-9282 (1989).
- Szwergold, B. S., Kappler, F., and Brown, T. R.,** Identification of fructose 3-phosphate in the lens of diabetic rats. *Science*, **247**, 451-454 (1990).
- Tabata, S., and Hizukuri, S.,** Studies on starch phosphates part 2. Isolation of glucose 3-phosphate and maltose phosphate by acid hydrolysis of potato starch. *Stärke*, **23**, 267-272 (1971).
- Takahashi, H., Yang, S. I., Kim, M., and Yamamoto, T.,** Protein and energy utilization of growing rats fed on the diets containing intact or partially hydrolyzed guar gum. *Comp. Biochem. Physiol.*, **107A** 255-260 (1994).
- Takeda, C., Takeda, Y., and Hizukuri, S.,** Structures of the amylopectin of amylomaize. *Carbohydr. Res.*, **246**, 273-281 (1993).
- Takeda, Y., and Hizukuri, S.,** Re-examination of action of sweet-potato beta-amylase on phosphorylated (1→4)- α -D-glucan. *Carbohydr. Res.*, **89**, 174-178 (1981).
- Takeda, Y., and Hizukuri, S.,** Location of phosphate groups in potato amylopectin. *Carbohydr. Res.*, **102**, 321-327 (1982).
- Takii, H., Yoshiyama, M., Yanase, M., Kamasaka, H., Nishimura, T., and Kuriki, T.,** The relationship of phosphoryl-oligosaccharides or sugarless chewing gum containing phosphoryl-oligosaccharides ingestion and gastrointestinal condition in humans: comparison between

men and women. *J. Appl. Glycosci. (in Japanese)*, **50**, 51-54 (2003).

Touati, E., and Danchin, A., The structure of promoter and amino terminal region of the pH 2.5 acid phosphatase structural gene (appA) of *E. coli*: a negative control of transcription mediated by cyclic AMP. *Biochimie*, **69**, 215-221 (1987).

Tsugawa, N., Yamabe, T., Takeuchi, A., Kamao, M., Nakagawa, K., Nishijima, K., and Okano, T., Intestinal absorption of calcium ascorbate in rats. *J. Bone Miner. Metab.*, **17**, 30-36 (1999).

Uchida, M., Kamasaka, H., Mathuura, T., Okada, S., and Ichikawa, T., Effect of phosphoryl oligosaccharides on calcium absorption in rats. *Oyo Toshitsu Kagaku (J. Appl. Glycosci.) (in Japanese)*, **43**, 535-540 (1996).

Ullah, A. H. J., and Cummins, B. J., Purification, N-terminal amino acid sequence and characterization of pH 2.5 optimum acid phosphatase (E.C. 3.1.3.2) from *Aspergillus ficuum*. *Prep. Biochem.*, **17**, 397-422 (1987).

Ullah, A. H. J., and Cummins, B. J., *Aspergillus ficuum* extracellular pH 6.0 optimum acid phosphatase: purification, N-terminal amino acid sequence, and biochemical characterization. *Prep. Biochem.*, **18**, 37-65 (1988).

Ullah, A. H. J., and Dischinger, Jr, H. C., Identification of active-site residues in *Aspergillus ficuum* extracellular pH 2.5 optimum acid phosphatase. *Biochim. Biophys. Res. Commun.*, **192**, 754-759 (1993).

- Ullah, A. H. J., and Phillippy, B. Q.,** Substrate selectivity in *Aspergillus ficuum* phytase and acid phosphatases using *myo*-inositol phosphates. *J. Agric. Food Chem.*, **42**, 423-425 (1994).
- Vihko, P., Virkunen, P., Henttu, P., Roiko, K., Solin, T., and Huhtala, M. L.,** Molecular cloning and sequence of cDNA encoding human prostatic acid phosphatase. *FEBS lett.*, **236**, 275-281 (1988).
- Vincent, J. B., Crowder, M. W., and Averill, B. A.,** Hydrolysis of phosphate monoesters: a biological problem with multiple chemical solutions. *Trends Biochem. Sci.*, **17**, 105-110 (1992).
- Wahlqvist, M. L., Wilmshurst, E. G., and Richardson, E. N.,** The effect of chain length on glucose absorption and related metabolic response. *Am. J. Clin. Nutr.*, **31**, 1998-2001 (1978).
- Watanabe, O., Hara, H., and Kasai, T.,** Effect of phosphorylated guar gum hydrolysate on increased calcium solubilization and the promotion of calcium absorption in rats. *Biosci. Biotechnol. Biochem.*, **64**, 160-166 (2000).
- Wennerholm, K., Arends, J., Birkhed, D., Ruben, J., Emilson, C. G., and Dijnman, A. G.,** Effect of xylitol and sorbitol in chewing gums on mutans streptococci, plaque pH and mineral loss of enamel. *Caries Res.*, **28**, 48-54 (1994).
- Wodzinski, R. J., and Ullah, A. H. J.,** Phytase. *Adv. Appl. Microbiol.*, **42**, 263-302 (1996).
- Wyss, M., Pasamontes, L., Friedlein, A., Rémy, R., Tessier, M.,**

- Kronenberger, A., Middendorf, A., Lehmann, M., Schnoebelen, L., Röthlisberger, U., Kusznir, E., Wahl, G., Müller, F., Lahm, H.-W., Vogel, K., and van Loon, A. P. G. M.,** Biophysical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): molecular size, glycosylation pattern, and engineering of proteolytic resistance. *Appl. Environ. Microbiol.*, **65**, 359-366 (1999a).
- Wyss, M., Brugger, R., Kronenberger, A., Rémy, R., Fimbel, R., Oesterhelt, G., Lehmann, M., and van Loon, A. P. G. M.,** Biophysical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): catalytic properties. *Appl. Environ. Microbiol.*, **65**, 367-373 (1999b).
- Yamamoto, T.,** in “Handbook of amylases and related enzymes”, eds. The Amylase Research Society of Japan, Pergamon Press, Oxford, pp40-44 (1988).
- Younes, H., Demigne, C., and Remesy, C.,** Acidic fermentation in the cecum increases absorption of calcium and magnesium in the large intestine of the rat. *Br. J. Nutr.*, **75**, 301-314 (1996).
- Zyla, K.,** Acid phosphatases purified from industrial waste mycelium of *Aspergillus niger* used to produced citric acid. *Acta. Biotechnol.*, **10**, 319-327 (1997).

LIST OF PUBLICATIONS

1. To-o, K., Kamasaka, H., Kusaka, K., Kuriki, T., Kometani, T., and Okada S., A novel acid phosphatase from *Aspergillus niger* KU-8 that specifically hydrolyzes C-6 phosphate groups of phosphoryl oligosaccharides. *Biosci. Biotechnol. Biochem.*, **61** (9), 1512-1517 (1997).
2. To-o, K., Kamasaka, H., Kusaka, K., Kuriki, T., and Okada S., Substrate selectivity in *Aspergillus niger* KU-8 acid phosphatase II using phosphoryl oligosaccharides. *Biosci. Biotechnol. Biochem.*, **64** (7), 1534-1537 (2000).
3. To-o, K., Kamasaka, H., Nishimura, T., Kuriki, T., Saeki, S., and Nakabou, Y., Bioavailability of calcium-bound phosphoryl oligosaccharides in rats. *J. Appl. Glycosci.*, **49** (2), 159-165 (2002).
4. To-o, K., Kamasaka, H., Nishimura, T., Kuriki, T., Saeki, S., and Nakabou, Y., Absorbability of calcium from calcium-bound phosphoryl oligosaccharides in comparison with that from various calcium compounds in the rat ligated jejunum loop. *Biosci. Biotechnol. Biochem.*, **67** (8), 1713-1718 (2003).
5. To-o, K., Inaba, D., Minami, K., Kamasaka, H., Nishimura, T., Kuriki, T., and Yonemitsu, M., Enhancing remineralization effect on enamel lesion by chewing gum containing phosphoryl oligosaccharides of calcium: human saliva immersing test by children volunteers.
In preparation