Mem. Fac. Agr. Kinki Univ. 32 : 1 ~19 (1999)

Research on Protopectinase : A New Aspect of Research on Pectolytic Enzymes

- Development of a Novel Microbial Function and Its Utilization in The Bioindustry -

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Synopsis

There are many ways to approach bioindustry, and one access may come from the observation of a particular behavior in nature. Microbiologists often search for the same kind of phenomenon in the protist kingdom, seeking way to apply the knowledge obtained through the interpretation of that natural behavior to the bioindustry.

This investigation was motivated by the increasing economical importance of an efficient utilization of agricultural and agroindustrial wastes. Sakai and his colleagues have paid much attention to the mechanism of breakdown of plant tissues, especially pectic substances that play a great roll on construction of tissues in the plant kingdom.

Pectic substances are prominent structural constituents of the cell wall in non-woody tissues. They exist as insoluble pectin, so called "Protopectin", and contribute for construction of plant tissues. They strongly influence the texture of vegetables and fruits. One of the most characteristic change of protopectin is observed during ripening: they become soft according to the solubilization of protopectin. The enzyme originally named protopectinase (PPase), which catalyses the solubilization of protopectin, has been assumed to be not exist. For a long time, PPase had been regarded as an enzyme that only macerates plant tissues, but little attention had been paid to its ability to liberate highly polymerized pectin (pectin-liberating activity).

Sakai and his colleagues researched this function in the protist kingdom, and they are now attempting to discuss thier discovery of this novel enzyme function (pectin-liberating activity) of PPases in relation to bioindustry.

I. Introduction

There are many ways to approach to a bioindustry. One access may start from the observation of a particular behavior in nature. Microbiologists, often, search the same kind of phenomenon in the protist kingdom, and seek the way to apply the knowledge obtained through the interpretation of that natural behavior, to the bioindustry.

Sakai and his colleagues have paid much attention to the mechanism of breakdown of pectic substances in the plant kingdom. Pectic substances are prominent structural constituents of the cell wall in non-woody tissues. In addition, they are the sole polysaccharides in the middle lamella mainly in the form of water-insoluble protopectin, which is responsible of cell cohesion (Fig. 1). They strongly influence on the texture of vegetables and fruits. One of the most characteristic change during ripening is softening. This process is attributed to the enzymatic

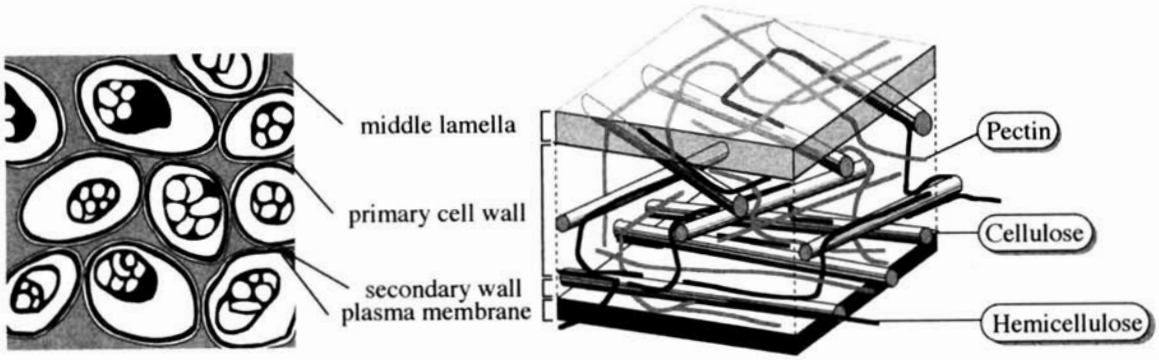


Fig. 1. Putative structure of plant tissues

degradation and solubilization of protopectin. The enzyme that catalyses the solubilization of protopectin was originally named protopectinase (PPase) by Brinton et al. in 1927 [1]. They proposed that this term should be applied to the enzyme that hydrolyzes or dissolves protopectin, causing plant cells to separate from each other, a process which is usually called maceration. During a long time, PPase had been regarded as an enzyme that only macerates plant tissues, but little attention was paid on its ability to liberate highly polymerized pectin (pectin-liberating activity). In 1978, Sakai and his colleagues started the research program by searching PPase activity in different microorganisms, and found a yeast that produced an enzyme showing pectin-liberating activity [2]. Thus, they suggested that protopectin was solubilized by restricted hydrolysis and proposed to call such kind of enzymes as "protopectinases".

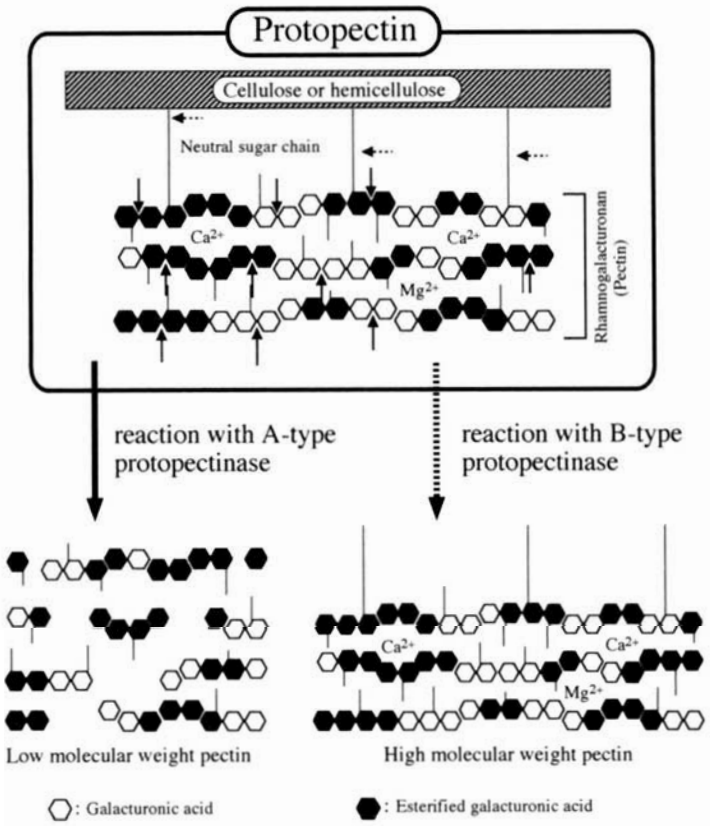


Fig. 2. Schematic illustration of protopectinase reaction

Since then, they isolated several PPases, which were classified into two types depending on their reaction mechanism. One type of PPases react with the polygalacturonic acid region of protopectin (smooth region) and the other type on the polysaccharide chains that connect the polygalacturonic acid chain to the other cell wall constituents (hairy region), as shown in Fig. 2. Sakai called the former A-type PPase and the latter B-type PPase [3]. We started the research on PPases motivated by the increasing economical importance of an efficient utilization of agricultural and agroindustrial wastes. Based on the results obtained, we have approached to the bioindustry.

Here, we will deal with PPases, their isolation, characterization, mechanism of pectin-releasing reaction, and utilization in the bioindustry.

II. A-type protopectinase

In 1978, the first study on pectin-liberating enzymes was reported by Sakai and Okushima [2]. A microorganism was detected that produced protopectin-solubilizing enzyme, which liberated water-soluble and highly polymerized pectin from protopectin. They also reported that protopectin is solubilized by restricted hydrolysis and called such enzymes "protopectinase". Since then, several PPases, which are classified into two types depending on their reaction mechanism, have been isolated [4-8].

Two types of A-type PPases are known; one has polygalacturonic acid hydrolyzing activity (A_1-type), and the other has polygalacturonic acid transeliminase activity (A_2-type).

II.1 A_1-type PPases

II.1.1 Occurrence of A_1-Type PPases

Some A_1-type PPases are found in the culture filtrate of yeasts and yeast like fungus. They have been isolated as crystals from culture filtrates of *Kluyveromyces fragilis* IFO 0288 (PPase-F)[6], *Galactomyces reessii* L (PPase-L)[5], and *Trichosporon penicillatum* SNO 3 (PPase-S) [4]; they are called PPase-F, -L, and -S, respectively.

Recently, such PPases have been isolated from the culture filtrate of some fungi, such as *Aspergillus awamori*, *Aspergillus niger* or *Trametes sunginea*.

Table 1. Physicochemical and biological properties of protopectinases

Properties	PPase-F	PPase-L	PPase-S
Molecular weight			
By electrophoresis	40,000	40,000	40,000
By gel filtration	33,000	30,000	30,000
By sedimentation	32,800	29,300	29,300
$s_{20,w}$	2.99S	3.77S	3.66S
$E^{1\%}_{280\,nm}$	10.0	11.9	9.20
Isoelectric point	5.0	8.4-8.5	7.6-7.8
N-terminal amino acid	Asp	Gly	Gly
Optimum pH	5.0	5.0	5.0
Optimum temperature (℃)	60	55	50
Inhibitor	Hg^{2+}, Hg^{+}, Ag^{+}, Ba^{2+}, Ca^{2+}, Pb^{2+}	Hg^{2+}, Hg^{+}, Ca^{2+}, Ba^{2+}, Co^{2+}	Hg^{2+}, Hg^{+}, Ca^{2+}, Ba^{2+}, Co^{2+}
pH stability	2-8	3-7	3-7
Activity (U/mg)			
Protopectinase	556	3,945	5,770
Polygalacturonase	2,053	16,219	21,107
Km value (mg/ml)			
For protopectin	90	50	30
For polygalacturonic acid[a]	6.6	7.7	9.0

[a]Polygalacturonic acid, having a mean polymethylization degree of 130, was used for the determination of the *Km* value.

II.1.2 Properties of the PPases

Some physical and biological properties of three PPases (PPase-F, -L, and -S) are shown in Table 1. These three PPases are similar in biological properties as well as in molecular weight, about 30,000, but not in specific activities. Table 2 shows the amino acid composition and carbohydrate content of PPases. Amino acid compositions of these enzymes are different. The antiserum to PPase-S gives precipitation lines with PPase-L and -S, but it does not react with PPase-F [8]. Amino acid sequences at the N-terminal and 27 residues long fragments in PPase-L and -S are identical (Fig. 3). PPase-F is not homologous to these two enzymes [8].

Table 2. Amino acid and sugar compositions of protopectinases

Amino acid	Amino acid residues[a] per molecule of protopectinase		
	PPase-F	PPase-L	PPase-S
Lysine	15	16	13
Histidine	4	6	5
Arginine	4	4	6
Tryptophan	5	9	5
Aspartic acid	40	35	37
Threonine	31	19	30
Serine	32	29	39
Glutamic acid	10	17	20
Proline	5	6	6
Glycine	30	42	34
Alanine	10	17	20
Half-cystine	4	1	6
Valine	13	18	16
Methionine	1	0	1
Isoleucine	13	20	21
Leucine	11	11	11
Tyrosine	4	4	5
Phenylalanine	7	10	8
Sugar	10[b]	3[b]	9[c]

[a]Calculations based on a molecular weight of 30,000.
[b]Determined as mannose.
[c]Determined as rhamnose.

II.1.3 Catalytic properties

The enzymes have pectin-releasing activity on protopectins from various origins. This is called PPase activity. The enzymes catalyze the hydrolysis of polygalacturonic acid: they decrease viscosity while slightly increasing the reducing value of reaction medium containing polygalacturonic acid [8]. Because of these findings, the enzymes are classified endo-polygalacturonases [EC 3.2.1.15; poly (1,4- α -D-galacturonide) glycanohydrolase].

The hydrolysis of galacturonic acid oligomers is change depend on the enzymes. Figure 4 shows the mode of action in the hydrolysis of galacturonic acid oligomers and gives the *K*m and *V*max values for the reaction. Three patterns of action toward galacturonic acid oligomers are known for endo-polygalacturonases [9~16]. PPase-S is novel in its action pattern towards oligogalacturonic acids. The *K*m and *V*max values change with the substrate chain-length; the *K*m values tend to decrease and the *V*max values tend to increase with increasing chain-length. *V*max is very different with trigalacturonic acid and tetragalacturonic acid. On the other hand, the number of methoxyl groups in the substrate affects the molecular weight of the reaction products; the molecular weight of the reaction products increases as the number of methoxyl groups in the substrate galacturonic acid increases (Fig. 5).

II.1.4 Postulated mechanism of A_1-type protopectinase activity

On the basis of the kinetic properties of the enzyme action as a polygalacturonase, the mechanism of PPase activity seems to be as follows: the enzyme reacts with the pectin molecule in protopectin at sites with three nonmethoxylated-galacturonic acid chains or more

PPase-F	D-S-G-T-L-S-G-K-T-A-G-G-G-L-S-N-?-A-T-V-T-V-N-N-V-?-V-P-A-G-
PPase-L	G-G-A-?-V-F-K-D-A-Q-S-A-I-A-G-K-A-S-?-?-S-I-T-L-Q-N-F-A-V-P-
PPase-S	G-G-A-?-V-F-K-D-A-Q-S-A-I-A-G-K-A-S-S-S-S-I-?-L-Q-N-F-
PPase-SE1	G-G-A-C-V-F-K-D-A-Q-S-A-I-A-G-K-A-
PPase-SE2	G-G-A-?-V-F-R-D-A-H-S-A-I-A-G-K-K-S
PPase-SE3	G-?-A-?-V-F-K-D-A-K-S-A-I-A-G-K-K

Fig. 3. N-terminal amino acid sequences of A_1-type PPases

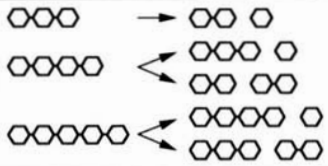
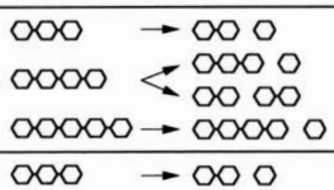
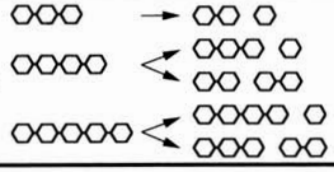
Enzyme	Substrates	Reaction products	*K*m (mM)	*V*max (μM/unit/min.)
Protopectinase-F			>10^2	<10^{-10}
			1.82	1.90×10^{-2}
			5.95×10^{-1}	2.95×10^{-1}
Protopectinase-L			3.98	1.96×10^{-3}
			2.77	2.37×10^{-2}
			7.09×10^{-1}	9.79×10^{-2}
Protopectinase-S			4.26	4.79×10^{-4}
			2.20	5.20×10^{-2}
			8.69×10^{-1}	2.85×10^{-1}

Fig. 4. Mode of action of protopectinases toward galacturonic acid oligomers

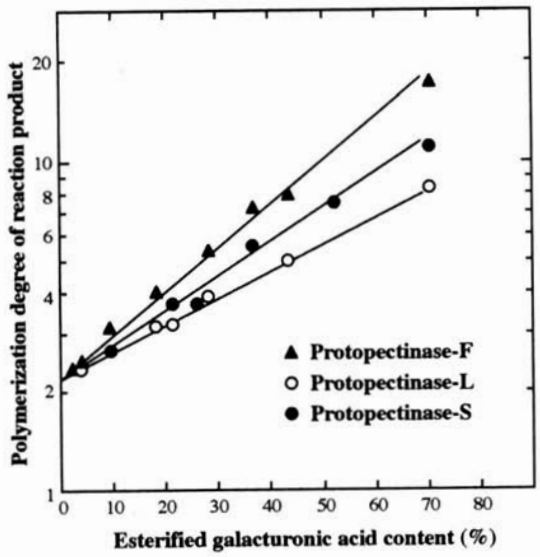


Fig. 5. Relationship between the amount of methoxyl groups of the substrate and the molecular weight of the degradation product of the enzyme reaction

(actually, four or more nonmethoxylated-galacturonic acid chains, considering the reaction velocities for galacturonic acid oligomers) and cleaves their glycosidic linkages. Generally, 50% of the galacturonic acids of pectin in protopectin are methoxylated, at random. Therefore, the pectin molecule in protopectin may be cleaved at restricted sites so as to form highly polymerized pectin. Furthermore, galacturonic oligomers inhibit the hydrolysis of soluble pectin but not of protopectin so that pectin released remains as highly polymerized form .

As mentioned above, these three PPases are a type of endo-polygalacturonase. Later, Sakai and Takaoka isolated an endo-polygalacturonase (polygalacturonase-AY) from the culture filtrate of *Aureobasidium pullulans*, which degrades polygalacturonic acid strongly but has weak PPase activity [17]. The enzyme has a lower affinity for

Table 3. Affinity of A_1-type protopectinases and polygalacturonase-AY on protopectin and polygalacturonic acid

Enzyme	*Km* values		Ratio of activity (Protopectinase/ Polygalacturonase)
	Protopectin[a] (mg/ml)	Polygalacturonic acid (mg/ml)	
PPase-F	90	6.6	0.33
PPase-L	30	9.0	0.24
PPase-S	50	7.7	0.23
PPase-SE1	55	7.9	0.23
PPase-SE2	—	—	0.15
PPase-SE3	—	—	0.18
Polygalacturonase-AY	525	3.8	0.03

[a]Obtained from *Citrus unshiu* peel.

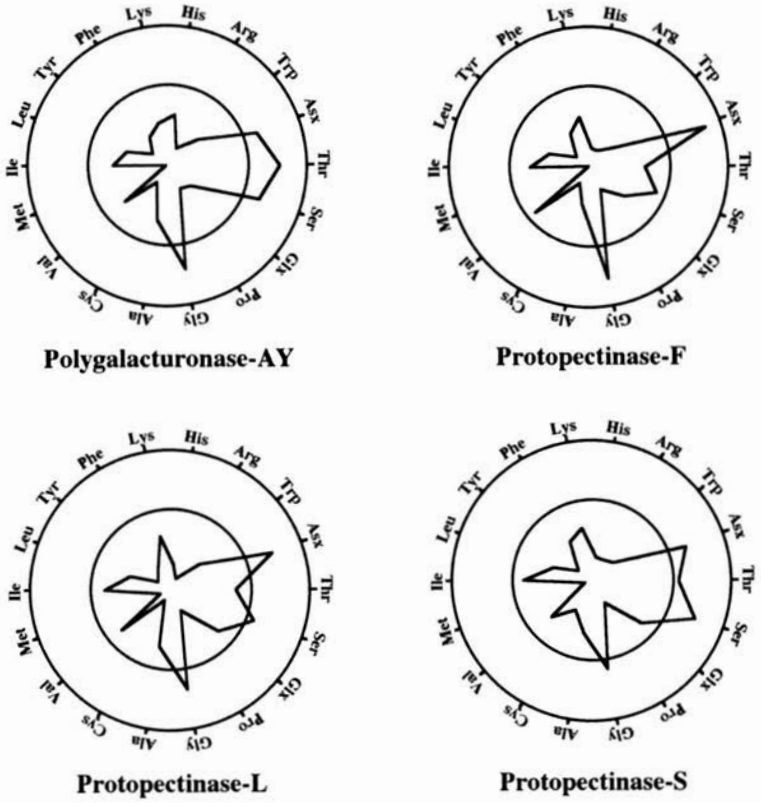


Fig. 6. Star diagrams of protopectinases and polygalacturonase-AY

protopectin from *Citrus unshiu* than PPase-F, -L, or -S; the *K*m value for protopectin is one order higher than those of the other three PPases, although they have almost the same affinity for polygalacturonic acid (Table 3). The differences in affinity for protopectin seem to be one reason why different endo-polygalacturonases have different PPase activity.

Thus, PPase activity is not common in endo-polygalacturonases, and only PPase-F, -L, and -S have been found to have very strong PPase activity. There are differences in their amino acid compositions, although the methionine content is low in all four enzymes. Polygalacturonase-AY contains more threonine than PPase-F, -L, and -S, and the star diagram of amino acid contents of polygalacturonase-AY is somewhat different from those of three PPases (Fig. 6) These facts may indicate that enzyme protein structure is very important factor to show PPase activity.

II.2 A_2-type PPases

Two A_2-type PPases have been found in the culture filtrate of strains belonging to *Bacillus* [18]. Those PPases, that split the glycosidic linkage of polygalacturonic acid (or methoxylated polygalacturonic acid) region in protopectin by transelimination reaction, have been isolated from the culture filtrate of *B. subtilis* IFO 3134. Those are named PPase-N and PPase-R, respectively. The former is active on polygalacturonic acid and the later has potent activity on methoxylated polygalacturonic acid.

II.2.1 Isolation of PPase-N and PPase-R

PPase-N and PPase-R are isolated from the culture filtrate of *B. subtilis* IFO 3134. Purification is done by column chromatographies using Butyl-Toyopearl 650M, CM-Toyopearl 650M, and Superose 12 and can be isolated as a homogeneous protein. Butyl-Toyopearl column chromatography gives a good separation of PPase-N from -R and -C (this strain also produced PPase-C and the details are described later). PPase-R and PPase-C are separated in a CM-Toyopearl column chromatography. EDTA inactivates

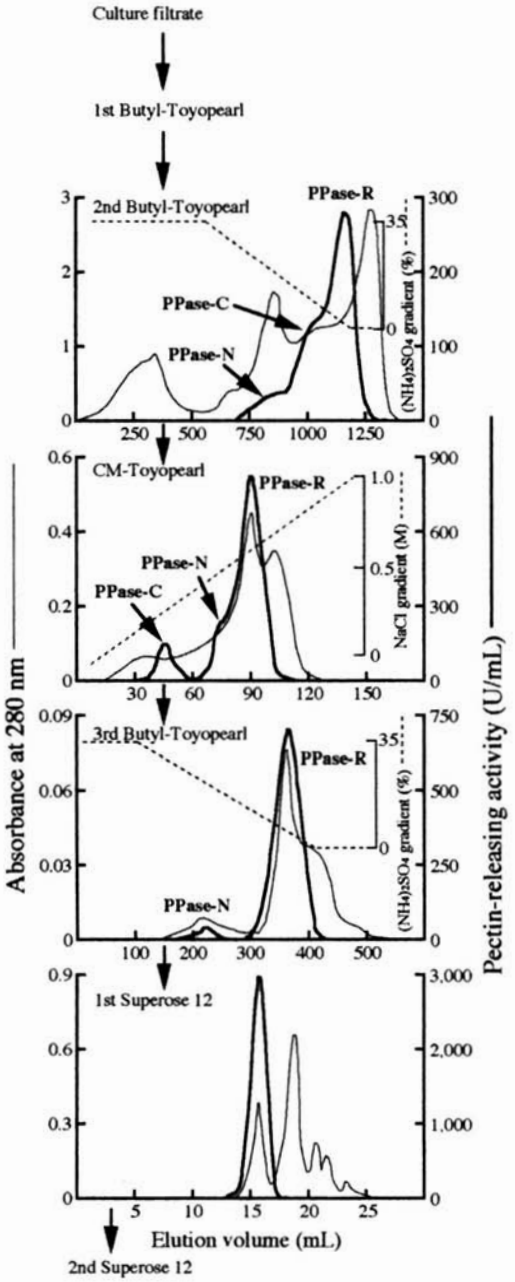


Fig. 7. Elution profiles of PPase-R on column chromatogtaphy during its purification

PPase-N but not PPase-R, so that measuring of PPase-R activity is carried out in the presence of 4 mM EDTA. The elution patterns on chromatographies of PPase-R are shown in Fig. 7.

II.2.2 Properties of PPase-N and PPase-R

Some physicochemical and biological properties of both PPases are summarized in Table 4. PPase-N is very susceptible to divalent cations and EDTA, but PPase-R is insensitive to EDTA.

Table 4. Some properties of PPase-N and PPase-R

Properties	PPase-N	PPase-R
Molecular weight		
SDS-PAGE	43,000	35,000
Gel filtration	32,000	27,000
Isoelectric point (pH)	9.4	8.2
Optimum pH (at 37℃)	8.0	8.0
Optimum temperature (pH 6.0)	60℃	60℃
Inhibitor	EDTA, Hg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Ba^{2+}	Hg^{2+}
pH stability (at 37℃ for 16hr)	3-10	4-11
Thermostability	~60℃	~60℃

Table 5. Activity of PPase-N and PPase-R toward polygalacturonic acid (PGA) with varying degrees of methyl esterification[a]

	Relative activity (%)	
Substrate	PPase-N	PPase-R
PGA	100	1
Methoxylated PGA (13% esterified)	97	6
Methoxylated PGA (26% esterified)	88	14
Methoxylated PGA (35% esterified)	80	25
Methoxylated PGA (58% esterified)	53	58
Methoxylated PGA (75% esterified)	14	100

[a]Methyl esterification of PGA was carried out according to the method of Jansen and Jang (1946). The methoxyl group in pectic acid is measured by the method of Wood and Siddiqui (1971).

These enzymes are stable in wide range of pH and temperature, and are different proteins by the criterion of immunological properties. N-Terminal amino acid sequences are also quite different. The substrate specificities of the enzymes towards polygalacturonic acid with various degrees of esterification are summarized in Table 5. Polygalacturonic acid is the best substrate for PPase-N, while PPase-R has potent activity on polygalacturonic acid with high degrees of esterification.

In the reactions, using polygalacturonic acid (or methoxylated polygalacturonic acid) or protopectin as the substrate, the enzymes produce the substance having absorption maximum at 235 nm, which may originate from the 4,5-unsaturated galacturonide (Fig. 8) [19].

With both enzymes, in the reaction on polygalacturonic acid or methoxylated

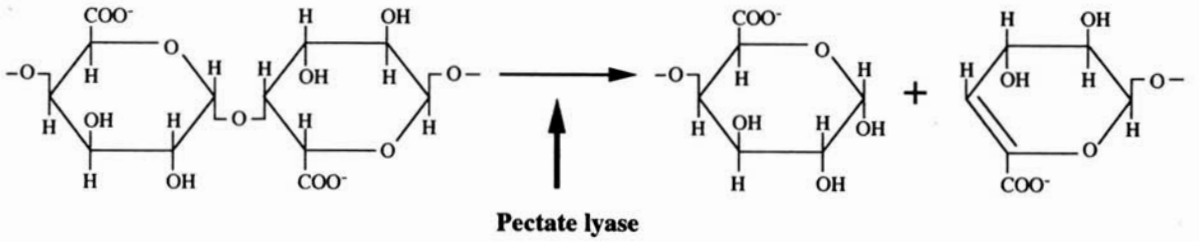


Fig. 8. Schematic illustration of pectate lyase reaction

polygalacturonic acid, relative viscosity of substrate solutions reduced over 50% when only a few percent of their glycosidic linkages are split. These data indicate that PPase-N and PPase-R split glycosidic linkage of pectic substances at random manner. Thus, PPase-N is an enzyme belonging to the category of pectate lyase [poly(1,4-α-D-galacturonide) lyase: EC 4.2.2.2] and PPase-R is an enzyme belonging to the category of pectin lyase [poly (methoxygalacturonide) lyase: EC 4.2.2.10], respectively.

III. B-type protopectinase

The reasons for the insolubility of the pectin in protopectin are complex, and include secondary valency bonding between pairs of pectin molecules or with other cell wall constituents such as cellulose or hemicellulose (Fig. 2) [20]. The features of the insolubility of the pectin in protopectin suggest that other enzymes - different from A-type PPases - have PPase-like activity, with a mechanism different from that involving the restricted hydrolysis of the polygalacturonic acid region in protopectin. Sakai et al. have found PPases which have a different mechanisms from A-type PPases.

III.1. Occurrence and isolation of B-type PPase

PPase that does not degrade polygalacturonic acid was first detected in the culture filtrate of *B. subtilis* IFO 3134 [21, 22]. The enzyme, called PPase-C, has potent activity on the protopectin from various origins. The same type of PPase (PPase-T) has found in the culture filtrate of microorganisms belonging to *Trametes* [23].

PPase-C is isolated from the culture filtrate of *B. subtilis* IFO 3134. The purification procedure consisted of the treatment of the culture filtrate with EDTA followed by chromatographies on CM-Cellulofine CH, Butyl-Toyopearl 650, and Toyopearl HW-55S.

PPase-T is purified from the culture filtrate of *T. sanguinea* IFO 6490 using column chromatographies on DEAE-Toyopearl 650, CM-Toyopearl 650, Butyl-Toyopearl 650, and Superose 12 (FPLC-GPC), and is isolated as a homogeneous preparation [23].

III.2 Properties of PPase-C and PPase-T

Although PPase-C and -T are B-type PPases, they have different molecular properties and substrate specificities. PPase-C has an apparent molecular weight of 30,000 (by SDS-PAGE) with an isoelectric point of around pH 9.0. Contrary to these, PPase-T is a protein having a molecular weight of 55,000 (by SDS-PAGE) with an isoelectric point of around pH 8.1. Some properties of PPase-C and -T are compared in Table 6. Amino acid compositions of the enzymes are also different; PPase-T are rich in aspartic acid, serine, and glycine, whereas PPase-C are rich in glutamic acid, serine, and glycine.

Table 6. Properties of PPase-C and PPase-T

Properties	PPase-C	PPase-T
Molecular weight	30,000	55,000
Isoelectric point (pH)	9.0	8.1
Optimum pH (at 37℃)	6.0	4.0
Optimum temperature (pH 6.0)	60℃	60℃
Inhibitor	Ag^+, Fe^{3+}, Hg^{2+}, Mn^{2+}, Sn^{2+},	Ag^+, Ca^{2+}
pH stability (at 37℃ for 16hr)	5-9	2-6
Thermostability	~60℃	~60℃

III.3 Activity of PPase-C and PPase-T on various protopectins

PPase-C and -T act on protopectin from various citrus fruit peels and other plant tissues, releasing pectin (Table 7). However, the pattern of pectin-releasing activity is different. PPase-C has high activity toward protopectins from sugar beet pulp and apple, but PPase-T has potent activity toward the protopectins from peels of Hassaku and Valencia orange, and carrot tuber, but less activity toward protopectins from sugar beet and apple. These results indicate that these two enzymes react with different sites in protopectin.

III.4 Reaction mechanism of PPase-C and PPase-T

The mechanism of the release of pectin from protopectin by the action of PPase-C and -T have been studied [24, 25]. In these studies, the smallest molecule polysaccharides serve as substrates of PPase-C and -T have been isolated from sugar beet pulp[26]. Using those polysaccharides as enzyme substrates, reaction mechanisms of B-type PPases were determined by comparing with structures of the substrates and the reaction products.

PPase-C catalyzes the degradation of α-1,5-L-arabinan and L-arabinan, which seems to have a main structure consisting of α-1,5-L-arabinan to which L-arabinofuranose units are attached at position 3 in the α-configuration to form one-unit side chains, from sugar beet pulp in a random manner, so it is an enzyme in the same category

Table 7. Comparison of activities of PPase-C and PPase-T[a]

Origin of protopectin	Relative activity (%)	
	PPase-C	PPase-T
Lemon peel (*Citrus limon*)	100	100
Hassaku peel	71	254
Satsuma mandarin orange peel (*C.unshiu*)	294	64
Valencia orange peel (*C.sinensis*)	550	174
Sugar beet pulp	2200	30
Apple	1300	49
Burdock (*gobou*) root	94	53
Carrot root	13	118
Radish tuber	419	74
Watermelon peel	0	87

[a]Reactions were done at 37℃, pH 6.0 (PPase-C) and pH 5.0 (PPase-T).

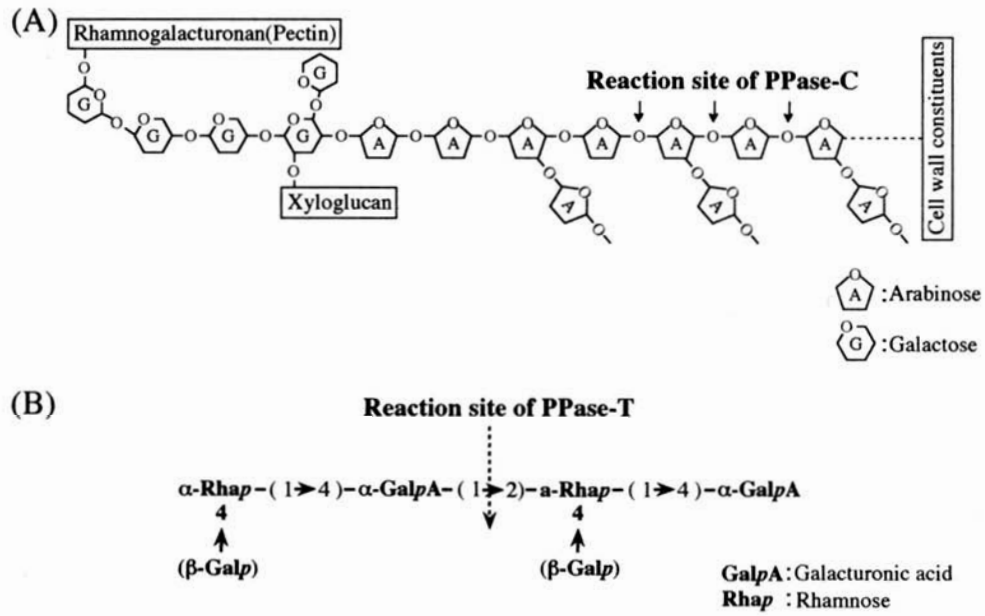


Fig. 9. Postulated structures of polysaccharides which are degraded with PPase-C (A) and PPase-T (B)

as arabinan endo-1,5-α-L-arabinase [EC 3.2.1.99]. However, endo-1,5-α-L-arabinase from *B. subtilis* (the enzyme catalyzes the hydrolysis of arabinooligomer to form L-arabinose and arabinobiose) shows low PPase activity. Thus, PPase-C is novel in the respect of having potent protopectinase activity[27].

As shown in Fig. 9, PPase-C splits the α-1,5-L-arabinofuranoside linkage of the arabinan region in arabinogalactan, which attaches pectin to the cell wall constituents, so that PPase-C releases pectin.

PPase-T can degrade rhamnogalacturonan in sugar beet protopectin, releasing pectic substance. The smallest polysaccharide (so called SPS) that can be the substrate of PPase-T was prepared from sugar beet pulp by the extraction with NaOH and digestion with α-L-arabinofuranosidase, α-L-arabinase, and β-1,4-D-galactanase. The reaction products of SPS with PPase-T are isolated by chromatographies on DEAE-Toyopearl 650M and Toyopearl HW40-S columns and analyzed by labeling of reducing ends with NaB^3H_4, and ^{13}C-NMR spectroscopy. The results indicated that PPase-T cleaves galactopyranosyluronic-rhamnopyranosyl linkages in SPS, so that PPase-T releases pectin (Fig. 9).

IV. Utilization of protopectinase

We have made much efforts to approach to the new bioindustry using PPases. Here, we will be dealing with some examples of utilization of PPases.

IV.1 In pectin production

Pectin is useful in the manufacture of food, cosmetics, and medicine; it is an industrially important substance, produced on industrial scale. Citrus peel, a by-product of the citrus processing industry, is a suitable source of pectin. In industrial production, pectin is extracted by placing the peel in vats of water, bringing the mixture to a boil as a slurry, and adding concentrated hydrochloric, sulfuric, nitric, or other acids to adjust the pH to about 2.0. Filtration of the extract is a tedious process because the extract, containing pectin and disintegrated peel, is both corrosive and viscous. The peel of mandarin orange (including *Citrus unshiu*, which accounts for more than 80% of the citrus fruit produced in Japan) is not suitable as a raw material in this process because the peel is fragile and becomes pasty when heated, which prevents the separation of pectin from the residues [28]. Thus, in Japan, pectin is not manufactured chemically, although nearly 5×10^5 tons of citrus peel, containing about 5% pectin on a fresh-weight basis, is produced each year.

Sakai and Okushima have tried pectin production with *C. unshiu* peel as the raw material by developing a new microbial method by which pectin can be enzymatically extracted from citrus peel without maceration of the peel [29]. The outline of the process is as follows; *T. penicillatum* SNO 3, which is a PPase-S producer, grows well in an extract of citrus peel as the sole nutrient source. The organism must assimilate the water-soluble carbon and nitrogen compounds in the peel, since nothing is present other than citrus peel. The amount of pectin extracted depends on the concentration of peel in the medium. The peel/water ratio of 1:2～1:3 is suitable. Pectin is extracted effectively between 25℃ and 30℃, although the microorganism grows well between 25℃ and 37℃. At 30℃, pectin begin to appear after 5 hours and the amount increases with fermentation time; after 20 to 25 hours, the amount of pectin extracted reaches a maximum. By this method, almost all of the pectin in the peel can be extracted. Physical and chemical properties of isolated pectin are compared in Table 8 with those of acid-extracted and commercial pectin. The pectin produced by this method is not very different from the two other kinds of pectin, except that this pectin contains more neutral sugar. Peels or segment covers of various citrus fruits are good raw materials; vegetables such as carrots, wax gourds, and radishes are poor as raw materials.

Based on these results, the new pectin bio-

Table 8. Some properties of pectin

Properties	Pectin type		
	From *Citrus unshiu* peel		
	Commercial (from lemon)	Extracted by fermentation	Extracted by acid-heat
Relative viscosity of 0.1% solution	1.53	1.46	1.23
Methoxyl group[a] (%)	9.24	8.58	9.13
Esterified carboxyl group[a]	63.1	73.8	66.1
Galacturonic acid[a] (%)	85.0	68.2	80.3
Neutral sugar[a] (%)	5.7	23.2	10.5
pH of 0.5% solution	3.96	3.24	4.34
Molecular weight[b] ($\times 10^{-3}$)	102	105	50
Element analysis[a]			
C	40.86	40.27	38.27
H	5.76	5.77	5.40
N	0.80	0.61	0.41

[a]Values expressed on an ash- and moisture-free basis.
[b]Calculated by the equation of Smit and Bryant (1967).

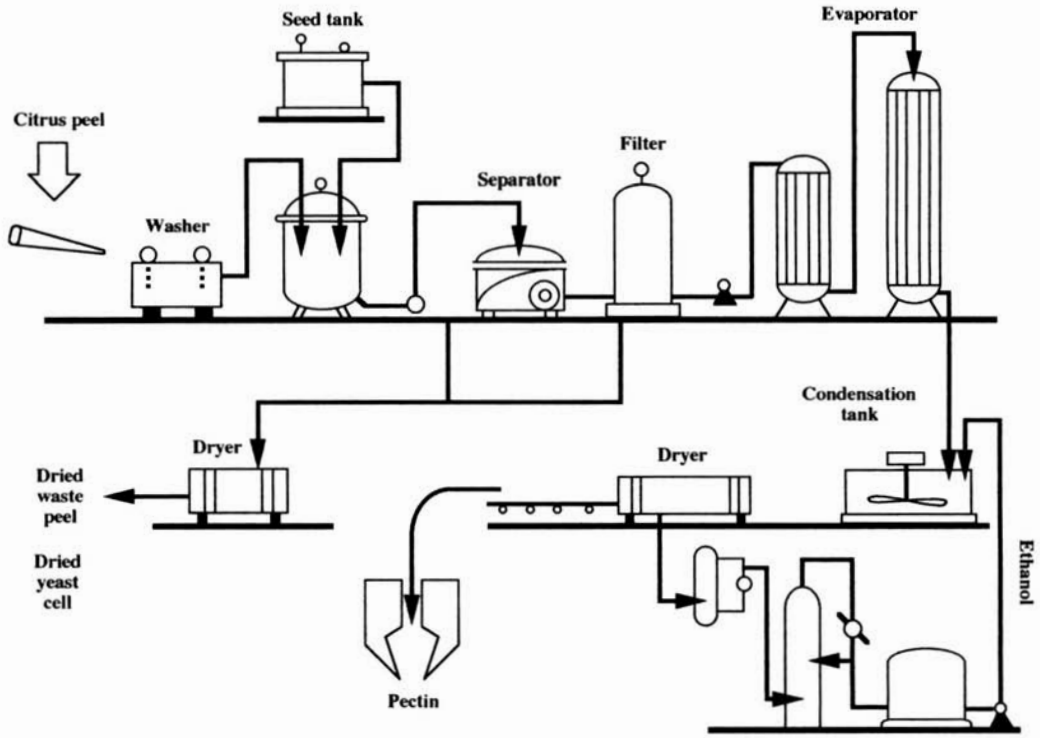


Fig. 10. Schematic diagram of the biochemical manufacturing process of pectin from citrus peel

production system established by Sakai and Okushima (Fig. 10) [30]. In this system, peels are washed, if necessary, and suspended in sterilized water in a fermentor, to which a seed culture of the microorganism is introduced (corresponding to from 3 to 5% of the volume of the fermentation broth) from a seed tank. After 15 to 20 hours of fermentation at 25 to 30℃, the residual peels are filtered off, and the resultant filtrate is passed through another filter to remove the microbial cells. The filtrate is concentrated and the pectin is precipitated in ethyl alcohol, collected, and dried. By this procedure, 20 to 25 kg of pectin is obtained per ton of mandarin peels, which are a by-product of the citrus processing industry.

IV.2. In preparation of plant protoplasts in biological experiment

PPases can be used for the isolation of plant mesophyll protoplasts. Mitsui et al. have shown

that PPase-S can be used for the isolation of mesophyll tissues of shoot such as *Tagetes minuta, Brassica rapa, Raphanus sativus, Lactuca sativa, Triticum aestivum, Secale cereale, Hordeum vulgare, Panicum crusgalli, Avena sativa, Zeamays* and *Oryza sativa* [31]. These plant cells often survive and regrown to produce a plant. PPase-S is outstanding for the preparation of protoplasts of monocotyledonous cells. The enzyme is obtained from commercial source, under the name of "Pectinase-GODO".

IV.3. In production of single-cell foods - a new food material

PPases are used for processing of single-cell preparations from vegetables for use in foods (Fig. 11). Vegetable cells isolated by enzymatic methods often survive because their biological functions are undamaged by such isolation procedure. Moreover, the flavors, pigments, spice substances, some kinds of bioactive peptides or nutrients such as vitamins etc. present in the original vegetable cells are likely to be preserved in single cells prepared in this way. Vegetables processed to give a single cell preparation might provide a new kind of ingredient for use. Nakamura et al. found that the PPases from *T. penicillatum, A. awamori*, and *B. subtilis* are useful for this purpose [32]. The constituents of the single cells are more stable than those in the preparation obtained by mechanical tissue disruption [33].

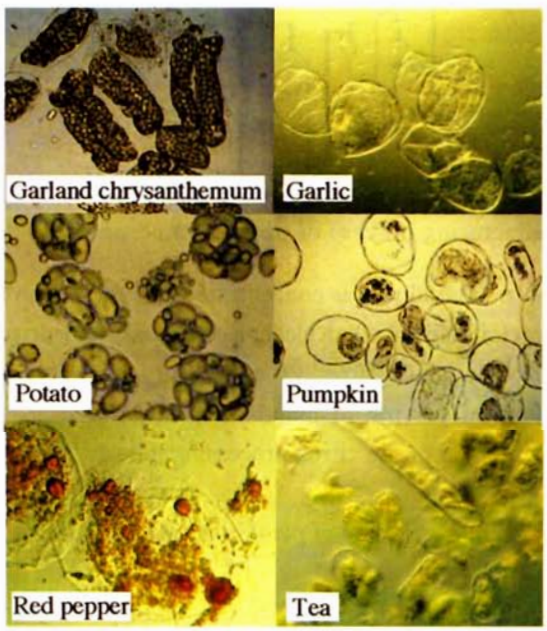


Fig. 11. Photomicrographs of the single cells obtained from different plant materials

II.4. In biochemical scouring of cotton fabric

Cotton fabric, which we intimately use in everyday life, is a natural fiber of cellulose constituents, with a structure of a primary wall and a secondary wall, the former covers the later as illustrated in Fig. 12. The primary wall, together with dirt, oil and other impurities (Table 9), is usually removed by a chemical process of scouring with sodium hydroxide. The process is well established on a commercial scale, already. However, as the chemical process is performed under strong alkaline condition (around pH 14 by boiling), the working condition is unsafe and wastes produced cause difficulties. To overcome these problems, Sakai and colleagues attempted to develop a biochemical process for scouring cotton fabric, and established a new process using PPases [34]. The new process has been named "BioScouring™" [35].

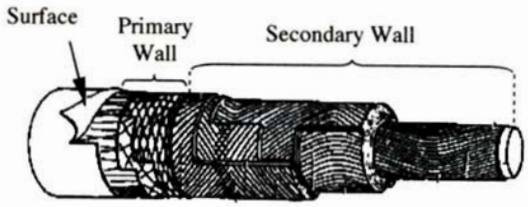


Fig. 12. Structure of cotton fiber

Table 9. Constituents in primary wall and fiber cotton

	Fiber (%)	Primary wall (%)
Cellulose	94.0	54
Protein	1.3	14
Pectic substance	0.9	9
Cotton wax	0.6	8
Ash	1.2	3
Others	-	4

In this process, PPases (PPase-N and/or -C) produced by *B. subtilis* is used, and we called the enzyme used in this process "Scourase". Operation of the process is quite simple; in the

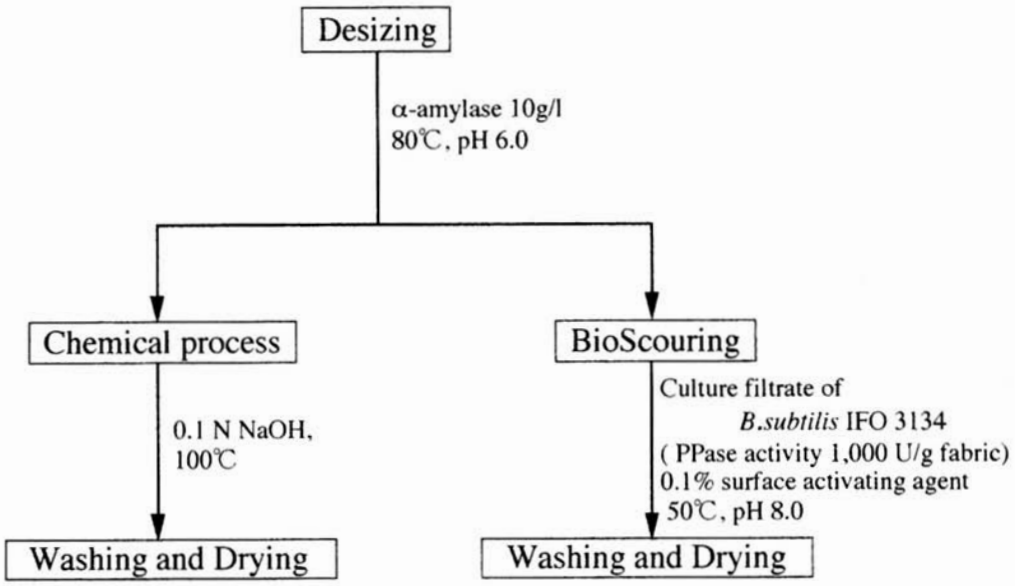


Fig. 13. Comparison of process for scouring of cotton fabrics

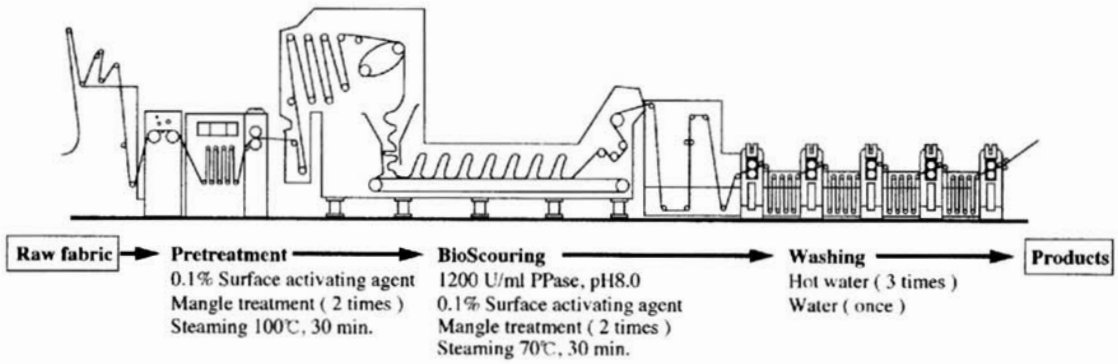


Fig. 14. Typical example of continuous BioScouring of fabrics

Table 10. Comparison of scouring process with various fabrics

Fabric		Pectin released (mg/g fabric)			Velocity of water absorption (cm/2min)[a]	
		Chemical scouring	BioScouring	Chemical scouring/ BioScouring (%)	Chemical scouring	BioScouring
Cotton 100%	: G169	13.1	12.6	96.2	4.2	5.8
Cotton 100%	: G81	23.8	21.7	91.2		
Cotton 35% + Tetron 65%	: G200	11.5	9.4	81.7	3.7	5.3
Cotton 35% + Tetron 65%	: G232	9.9	8.9	89.9		
Cotton 100%	: TM 2710	6.4	8.1	126.6	9.6	8.6
Cotton 100%	: SK 87603	6.7	8.5	126.9		
Cotton 50% + Polyester 50%	: TM 3010	3.3	4.4	133.3	9.4	9.5
Cotton 50% + Polyester 50%	: SK 92026	3.2	4.1	128.1		

[a]Determined by rising velocity of Eosin solution.

industrial process, raw cotton fabric is incubated in a solution containing sufficient amounts of PPases and small amount of surface-active agent, pH 8.0, at 50℃ for 30 min (Fig. 13). Figure 14 illustrates a typical industrial equipment used for continuous BioScouring process. By this process, about 80% of pectic substances in the raw cotton fabric are removed and to obtained high quality of fabric on the criterion of water absorbency quality. This process is applied for scouring not only sole cotton fabric but also such mixed spun fabrics as cotton-wool or cotton-chemicals (Table 10).

BioScouring has the following merits over the conventional scouring method with caustic soda: (1) retains the fiber strength less degenerated, (2) gives the fabric a much softer feel, (3) allows for a safer working environment, (4) creates much less environmental pollution, and (5) lowers energy cost [35-38].

V. New trends in protopectinase research

Researches on PPase have been motivation to open a new ear in research of biochemistry.

Here we will be dealing the new research problem motivated by PPase research.

V. 1 The study on ultra acid stable enzymes (UAS enzyme)

There is an intense relationship between enzymatic activity and quaternary sturcture of enzyme protein. Although, there are some specific enzymes are known to be stable at extremely abnormal conditions, such as strong alkaline condition (alkaline enzyme) or high temperature (thermotorelant enzyme), generally, enzymes lose their catalytic activities under such extreme conditions, as high temperature, high salt concentration, extremely high or low pH.

In the course of research on PPase, we have found that some strains belonging to *Aspergillus* produce the enzymes enduring in strong acidic condition [39, 40]. We called such enzyme Ultra Acid Stable (UAS) enzyme, a criterion of maintaining the initial activity after incubation at pH 2.0, 37℃ for 10 hours. We have found two UAS enzymes, so far, protopectinase-AS (PPase-AS) [41] and arabinofuranosidase-AU (ABF-AU), from *A. awamori* and and *A. usamii* mut. *shiro-usamii*, respectively [42].

PPase-AS, produced by *A. awamori* a A_1-type PPase, is most active at around pH 2.0 but not active at pH 5.0. The enzyme is most stable at pH 2.0: the enzyme retains initial activity after incubation at pH 2.0, 37℃ for 10 hours, while losing the activity incubating at pH 5.0, 37℃ for 10 hours, completely (Fig. 15). We isolated another A_1-type PPase, PGase-X2, produced by

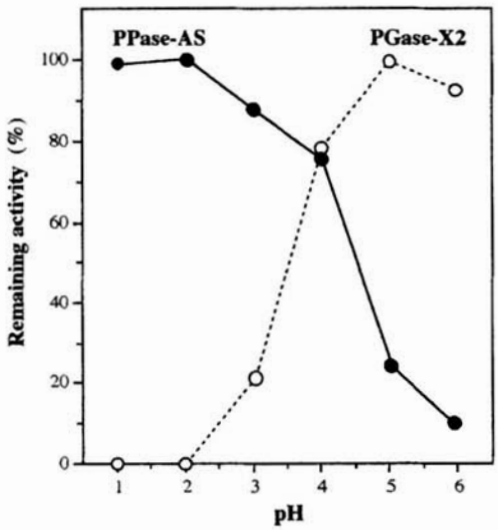


Fig. 15. Effect of pH on enzyme stability

the same strain [43]. PGase-X2 and PPase-AS have similar properties except for stability at acidic condition; the enzyme loses its activity when incubated at pH 2.0 (Fig. 15).

Another UAS enzyme, arabinofuranosidase-AU, was isolated from the culture filtrate of *A. usamii* mut. *shiro-usamii*. The strain produces an arabinofuranosidase, acid-unstable-enzyme

Table 11. Physicochemical and biological properties of arabinofuranosidases

Property	ABF-AU	ABF-AL
Molecular weight		
By electrophoresis	65,000	65,000
By gel filtration	65,000	65,000
N-terminal amino acid	Glycine	-
Optimum pH	2.0-5.0	4.5
Optimum temperature (℃)	37	60
pH stability	2.0-5.0	2.5-5.5
Activity (U/mg)	115.3	1.42
Km value (mM)	17.3	-
pI	3.0	-

arabinofuranosidase (ABF-AL) besides ABF-AU. These two ABF have similar properties except for stability in acidic condition; ABF-AU is stable at pH 2.0, on the other hand, ABF-AL loses its activity at pH 2.0 completely (Table 11, Fig. 16). Production of these two arabinofuranosidases are dependent on ambient pH [44]; at pH 2.0, ABF-AU is produced but not ABF-AL, and the opposite phenomenon is observed at pH 5.0.

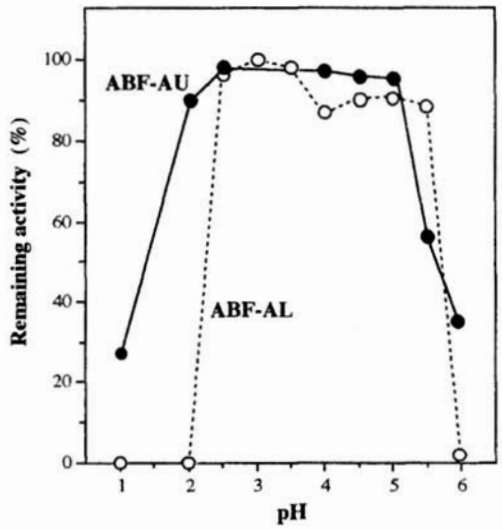


Fig. 16. Effect of pH on enzyme stability

Concerning pH-regulation of gene expression, there are some studies on gene expression in *A. nidulans* and *A. niger*, which are mediated by the wide-domain zinc-finger transcription factor PacC [45, 46]. In alkaline culture conditions, this factor is converted to its truncated functional form in response to ambient pH signal transduced by the products of *pal* genes, and is able to active the expression of those genes whose products are appropriate at alkaline ambient pH and repress those whose expression is

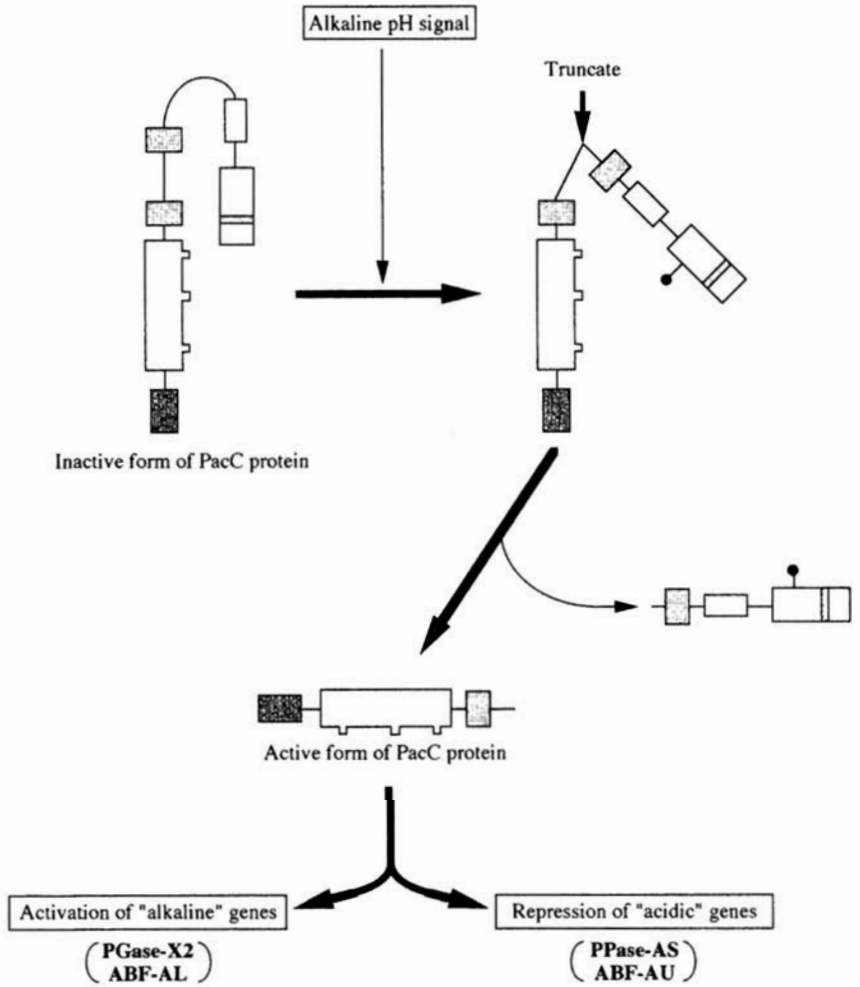


Fig. 17. Putative model for the role of PacC proteolysis in pH regulation (from Orejas et al. [48] with some modifications)

suited to acidic pH (Fig. 17)[47, 48]. Such a regulation system of gene expression mediated by PacC may exist in our strains. We cloned a gene encoding polygalacturonase (PGase-X2, *pgx2* gene) from *A awamori* and investigated its structure, and confirmed that the gene contained consensus sequence of PacC binding site [49]. From the facts, we suppose that promoter of PPase-AS gene may contain the sequence for PacC binding site. However, details of the mechanism on the regulation system of pH-mediated gene expression, remains to be resolved. Because few microorganism can grow at pH 2.0, those enzymes stable at that pH, UAS enzymes, are useful for industrial purposes.

Thus, these enzymes seem to be favorable not only for industrial purposes but also as a material for research in biochemistry. Such studies could include the relation between acid-stability and the molecular structure of enzyme protein and the regulation of gene expression.

V. 2 Research on the molecular structure of PPases

As mentioned in I.1.4, protopectinase activity of A-type PPase is a unique properties of PGases (or polymethylgalacturonase). Sakai *et al.* have proposed to indicate the degree of protopectinase activity of A-type PPase by an index, so call *q*-value [50]. Namly, a ratio of activity on protopectin *vs.* activity on polygalacturonic acid:

$$q\text{-value} = \text{activity on protopectin} \,/\, \text{activity on polygalacturonic acid (or on polymethylgalacturonic acid)}$$

Iguchi *et al.* have isolated three kinds of PPases, PPase-SE1, -SE2 and -SE3, from a culture filtrate of *T. penicillatum* B2, which is a γ-ray irradiation mutant induced from *T. penicillatum* SNO 3. These three enzymes have different *q*-values, although their homologies in primary structure are higher than 95% (Table 3, 12) [50]. These facts may indicate that protopectinase activity of endo-polygalacturonase is dependent on its stereo-structure. We presumed that the differences in protopectinase activity of A-type PPases is due to their molecular structure. But the three-dimensional structure of above PPases has not yet been resolved. Determination of the three-dimensional structure of A-type PPases gives insight into the relation between the molecular structure of the PPases and protopectinase activity.

The relation between the molecular structure of A_2-type PPase and protopectinase activity is also being investigated. We have investigated on multiplicity of A_2-type PPase in the strains belonging to *Bacillus*, using immunological and Southern hybridization analysis of these genomic DNAs. As the result, their A_2-type PPase are divided into three groups: tentatively called C-, G- and N- group, respectively. Their *q*-values were identical, although their pH stability and thermostability were different. On the other hand, we isolated a thermostable polygalacturonate (PGA) lyase having protopectinase activity produced by a thermophilic bacillus. Its *q*-value was much lower than that of A_2-type PPase from above mesophilic bacilli. The difference in *q*-values suggests that the molecular structures of these enzymes are different.

Recently, the three dimensional structures of PGA lyase (PelC) from *Erwinia chrysanthemi* EC16 was determined [51, 52]. PelC, one of the gene products from the pelBC gene family, believed to be an essential virulence factor in pathogenesis. The enzyme have a unique structure, the "parallel β-helix", which is generated by coiling a β- strand into a large, right-handed helix with an unusual stacking of asparagines on consecutive turns of the parallel β-helix core. Kita et al.

Table 12. The *q*-values of three PPases from *T. penicillantum* B2

Origin of protopectin	*q*-values (PPase activity/PGase activity)		
	PPase-SE1	PPase-SE2	PPase-SE3
Lemon	1.18	0.18	0.93
Orange	0.06	0.02	0.01
Grapefruit	0.24	0.06	0.08
Sugar beet pulp	0.98	0.3	0.39
Carrot	0.09	0.10	0.19
Potato	0.31	0.07	0.01
Apple	0.09	0.03	0.05

constructed several oligonucleotide site directed PelC mutant proteins. Some of them exhibit higher *q*-value (plant tissue maceration activity / PGA lyase activity) than that of wild-type PelC [53]. On the other hand, Pickersgill et al. determined the three dimensional structures of PGA lyase from *B.subtilis* SO 113 [54]. The enzyme has right-handed parallel β-helix structure (same as PelC) although *B. subtilis* A_2-type PPase has low homology with PelC (32% amino acid identity in mature region). PPase-N has significant homology (98% amino acid identity in mature region) with PGA lyase from *B.subtilis* SO 113, therefore PPase-N would have parallel β-helix structure. In addition to above PGA lyase, other examples of right-handed parallel β-helix were observed in the structure of *A. niger* pectin lyase A and *A. aculeatus* rhamunogalacturonase A [55, 56]. It is unknown whether these pectolytic enzymes involves right-handed parallel β-helix structure for protopectinase activity (or maceration activity) on protopectin.

We are currently working on the gene cloning and molecular structures of A_2-type PPases.

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プロトペクチナーゼに関する研究：ペクチン分解酵素の研究の新展開

— 微生物の新機能の開発とそのバイオインダストリーへの利用 —

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要　　約

バイオインダストリーを設立する手法は様々であるが、自然界における特異的な現象に注目し、その現象をバイオインダストリーに結びつける場合がある。微生物学の研究者は、自然界における特異的な現象を微生物界に検索し、その特異現象の研究で得た知見を応用してバイオインダストリーに結びつけることを試みる場合がしばしばある。

この研究は、農業・農産廃棄物を有効に利用することの経済的な重要性に触発されて開始したものであるが、坂井らは植物組織中のペクチン質の分解機構に注目した。

ペクチン物質は非木質植物組織の細胞壁中に最も多く存在する物質で、植物組織中では不溶性のプロトペクチンとして存在し、野菜や果物の食感に大きな影響を与える物質であることが知られている。さて、果物や野菜類が熟化する過程での最も大きな変化は軟化することにあるが、その際プロトペクチンが分解する。プロトペクチンを分解する酵素としてプロトペクチナーゼと名付けられた酵素の存在が推測されていた。永い間、プロトペクチナーゼは植物組織を崩壊させる酵素として認識され、プロトペクチンから高分子のペクチンを可溶化する機能については全く顧みられなかった。坂井らは、プロトペクチンを分解する酵素をプロトペクチンから高分子のペクチンを遊離させる機能を持つ酵素として微生物に見出し、この新規機能をバイオインダストリーに結びつけることを試みた。

この総説では、それらの研究の概略を紹介する。