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Short communication

Cloning and Expression of Alkaline Phosphatase Gene from *Schizosaccharomyces pombe*

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A cDNA coding alkaline phosphatase (AP) homologue was isolated from a cDNA library of *Schizosaccharomyces pombe* by colony hybridization. The nucleotide sequence of the cloned cDNA appeared to lack the N-terminal coding region. The genomic DNA encoding alkaline phosphatase homologue was isolated from *S. pombe* chromosomal DNA using PCR. The amplified DNA fragment was ligated into plasmid pRS315 to generate the recombinant plasmid pSW20. The DNA insert was subcloned as two smaller fragments for nucleotide sequencing. The sequence contains 2,789 bp and encodes a protein of 532 amino acids with a molecular mass of 58,666 daltons. The *S. pombe* cells containing plasmid pSW20 showed much higher AP activity compared with the yeast cells with vector only. This indicates that the cloned AP gene apparently encodes AP. The predicted amino acid sequence of the *S. pombe* AP shares homology with those of other known APs.

Keywords: Alkaline phosphatase, cDNA, Fission yeast, Genomic DNA, *Schizosaccharomyces pombe.*

Introduction

Alkaline phosphatases (APs; EC 3.1.3.1) are dimeric, metal-containing nonspecific phosphomonoesterases that hydrolyze a broad range of monophosphates at alkaline pH. APs in general are present in a broad diversity of eukaryotic and prokaryotic organisms, suggesting that the nonspecific hydrolysis of phosphate esters is functionally important. The AP reaction proceeds through a phosphoenzyme intermediate. AP cDNAs or genomic DNAs were cloned from a variety of species, such as the silkworm *Bombyx mori* (Itoh *et al.*, 1991), the cyanobacterium *Synechococcus* sp. (Wagner *et al.*, 1995), rat intestine (Lowe *et al.*, 1990), bovine intestine (Manes *et al.*, 1998), *Zymomonas mobilis* (Gomez and Ingram, 1995), *Bacillus subtilis* (Hulett *et al.*, 1991), and the budding yeast *Saccharomyces cerevisiae* (Kaneko *et al.*, 1987).

AP purified from the bacterium *Prevotella intermedia* contains phosphotyrosyl phosphatase activity (Ansai *et al.*, 1998). AP from *S. cerevisiae* was demonstrated to possess phosphoprotein phosphatase activity on the phosphoseryl proteins histone 11-A and casein, suggesting that the physiological role of the p-nitrophenyl phosphate-specific phosphatase may involve participation in reversible protein phosphorylation (Tuleva *et al.*, 1998). The two isozymes of rat intestinal APs differ in their primary structure, substrate specificity, tissue localization, and response to fat feeding (Xie and Alpers, 2000). Nitric oxide plays a regulatory role in AP activity during rat fracture healing (Namkung-Matthai *et al.*, 2000).

The expression of various AP genes is regulated in response to the inorganic phosphate concentration in the medium, suggesting that the enzyme functions to provide a source of inorganic phosphate. Transcription of the genes encoding AP, and the inorganic phosphate transporter of *S. cerevisiae*, are coordinately repressed and de-repressed, depending on the inorganic phosphate concentration in the culture medium (Oshima *et al.*, 1996). Transcription factor Sp3 was reported to activate the liver/bone/kidney-type alkaline phosphatase promoter in hematopoietic cells. Here, as a preliminary step in the study on *Schizosaccharomyces pombe* AP, we describe the cloning and expression of its cDNA and genomic DNA.

Materials and Methods

Chemicals Ampicillin, p-nitrophenyl phosphate, and N-lauroylsarcosine were purchased from Sigma Chemical Co. (St. Louis, USA). Restriction enzymes (*Eco*RI, *Xho*I, *Hind*III, and *Bam*HI), T4 DNA ligase, RNase, proteinase K, PCR core kit, and DIG (digoxigenin) high prime labeling and detection starter kit I

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were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Seakem LE agarose and nitrocellulose membrane (Trans-Blot Transfer Medium) were from BioProducts (Maine, USA) and Bio-Rad Laboratories (Hercules, USA), respectively. The *S. pombe* MATCHMAKER cDNA library was purchased from Clontech Laboratories, Inc. (Palo Alto, USA). The 5'-DIG labeled DNA probe (5'-GCGGTTGCTCCGGCTGCACTATCCG-3') and the two PCR primers (primer 1, 5'-CCTGGATGGTTAGGGATCC CCAAATGT TAAT-3'; primer 2, 3'-AGGTAATCGCGGCTGCA GTGGATGATAT-3') were purchased from TaKaRa Shuzo Co. Ltd., Japan.

Strains and growth conditions The cDNA library was constructed in the *Escherichia coli* strain DH10B, and was amplified by growing it in a LB medium containing 50 μg/ml ampicillin. *S. pombe* KP1 (h^+ *leu1-32 ura4-294*) was used for transformation. The yeast cells were grown at 30°C with shaking. The cell growth was monitored by the absorbance at the wavelength of 600 nm (Cho *et al.*, 2000a).

Cell harvest and disruption The yeast cells were harvested by centrifugation. They were frozen and resuspended in a 20 mM Tris buffer (pH 8.0)/2 mM EDTA (buffer A) and disrupted by using a glass bead beater and a sonicator. Supernatant was obtained after centrifugation and used as a crude extract for enzyme assays (Kim *et al.*, 1999; Cho *et al.*, 2000b).

Colony hybridization To screen the cDNA encoding alkaline phosphatase from the *S. pombe* cDNA library (Clontech Laboratories, Inc.), colony hybridization was performed according to the procedure described in 'The DIG System User's Guide for Filter Hybridization' produced by Roche Molecular Biochemicals. The summarized procedure is as follows: Colonies on the agar plate were pre-cooled for approximately 30 minutes at 4°C, and a membrane disc was carefully placed onto the surface. The transferred DNA was then cross-linked by baking the dry membrane for 1 h at 80°C. It was next placed on a clean piece of aluminum foil and treated with proteinase K. The membrane disc was pre-hybridized for 1 h in a pre-hybridization solution. After the pre-hybridization solution was discarded, the disc was placed in a hybridization solution containing 5'-DIG labeled DNA probe. The hybridization reaction was conducted at 65°C for about 15 h. After hybridization and washing, the disc was subjected to colorimetric detection with NBT and BCIP.

Southern hybridization Plasmid DNA was digested with the appropriate restriction enzymes, and separated on a 0.8% agarose gel. The separated DNA fragments were blotted according to the vacuum blotting procedure in 'Instruction Manual of Vacuum Blotter' (Bio-Rad Laboratories). Hybridization and colorimetric detection were conducted according to the procedures used in colony hybridization.

PCR PCR was performed as described in the user's sheet offered by Roche Molecular Biochemicals. The PCR conditions used in this study was 98°C (10 sec), 57°C (30 sec), 72°C (3 min) for 30 cycles.

Nucleotide sequencing The nucleotide sequencing was performed with an automatic DNA sequencer in Bionex, Inc., Korea. The determined nucleotide sequence reported in this study has been submitted to the GenBank database under the accession number AF316541.

Enzyme assay AP activity was determined as previously described (Harb *et al.*, 1991). The hydrolysis of p-nitrophenyl phosphate at pH 8.0 was monitored at 405 nm. Specific activity was expressed in the ΔA_{405}/min/mg protein. The protein concentration was determined according to the procedure of Bradford (1976) using bovine serum albumin as a standard.

General techniques The other recombinant DNA techniques used in this study were performed according to 'Molecular Cloning: A Laboratory Manual' (Sambrook *et al.*, 1989).

Results and Discussion

The fission yeast *S. pombe* is known to be very similar to higher eukaryotic organisms in the aspects of physiology and genetics. It is highly acceptable for the production of pharmaceutical proteins expressed from recombinant DNAs. There is not much information available on AP of *S. pombe*. However, the plausible nucleotide sequence encoding *S. pombe* AP homologue was stored in the GenBank database.

cDNA A *S. pombe* cDNA library, constructed in the vector pGAD GH, was purchased (Van Aelst *et al.*, 1993). The *Eco*RI/*Xho*I site of the vector was used for library construction. The 5'-DIG labeled DNA probe was prepared as described in 'Materials and Methods'. Colony hybridization yielded one positive cDNA clone for *S. pombe* AP. Plasmid DNA was purified from the cDNA clone and subjected to Southern hybridization to confirm the AP cDNA. The DNA probe, which had been used in colony hybridization, was also used in Southern hybridization. The plasmid DNA containing *S. pombe* AP cDNA was named pSW10. Plasmid DNA pSW10 was digested with *Eco*RI and *Xho*I, and the insert fragment was transferred into the shuttle vector pRS316 to generate plasmid pSW11. The recombinant plasmid pSW11 DNA harboring *S. pombe* AP cDNA was subjected to automatic DNA sequencing. Since the shuttle vector pRS316 contains T7 and T3 promoter sequences at the both sides of the multiple cloning site, the two strands of the insert DNA were possibly sequenced from the recombinant plasmid pSW11. The determined cDNA sequence was shown in Fig. 1. The cDNA clone contains a 1,696 bp insert, and has a unique reading frame of 502 amino acids. However, it contains no N-terminal 30 amino acids when the coding region is compared with the sequence information stored in the GenBank database. Since the *Eco*RI restriction site was used for the construction of the cDNA library, the unique *Eco*RI site in the coding region of *S. pombe* AP was digested during the construction, and it resulted in the loss of the N-terminal region. Although the *S. pombe* AP cDNA does not have the whole coding region, the presence of the cDNA

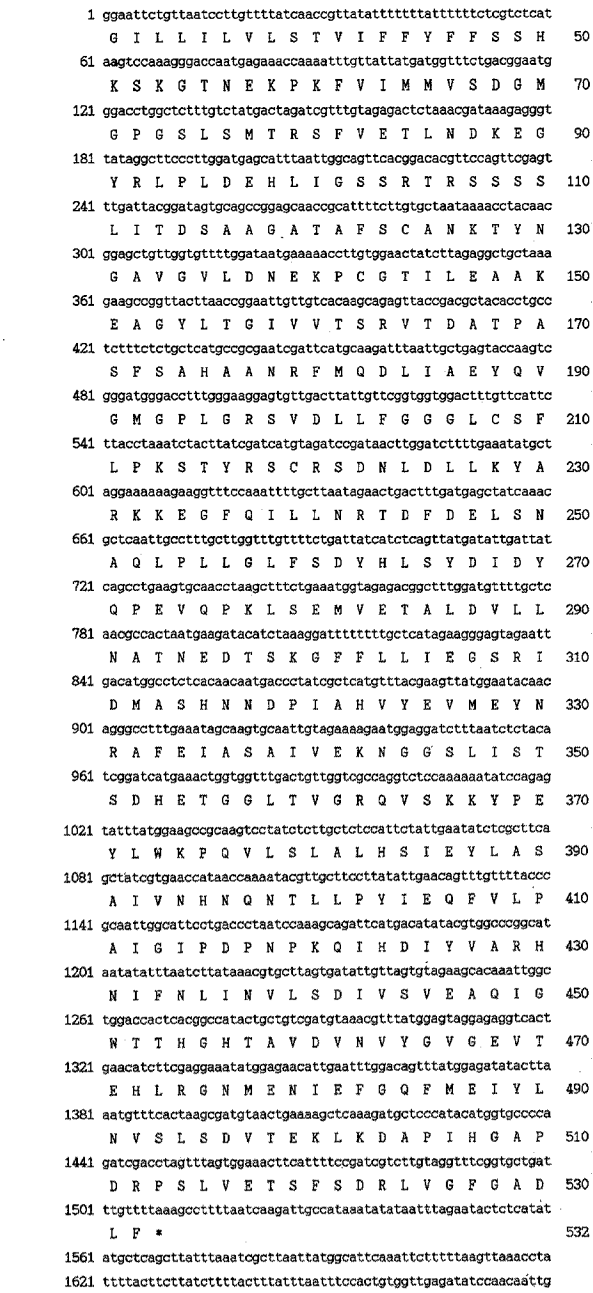

Fig. 1. The nucleotide sequence and deduced amino acid sequence of partial cDNA encoding alkaline phosphatase from *S. pombe*. The nucleotides are numbered from 5'-3', and their numbers are marked in the left margin. The numbers of amino acids in the putative alkaline phosphatase are marked in the right margin. The N-terminal 30 amino acids are deleted in the shown sequence. The asterisk indicates the stop codon. A poly A tail is underlined.

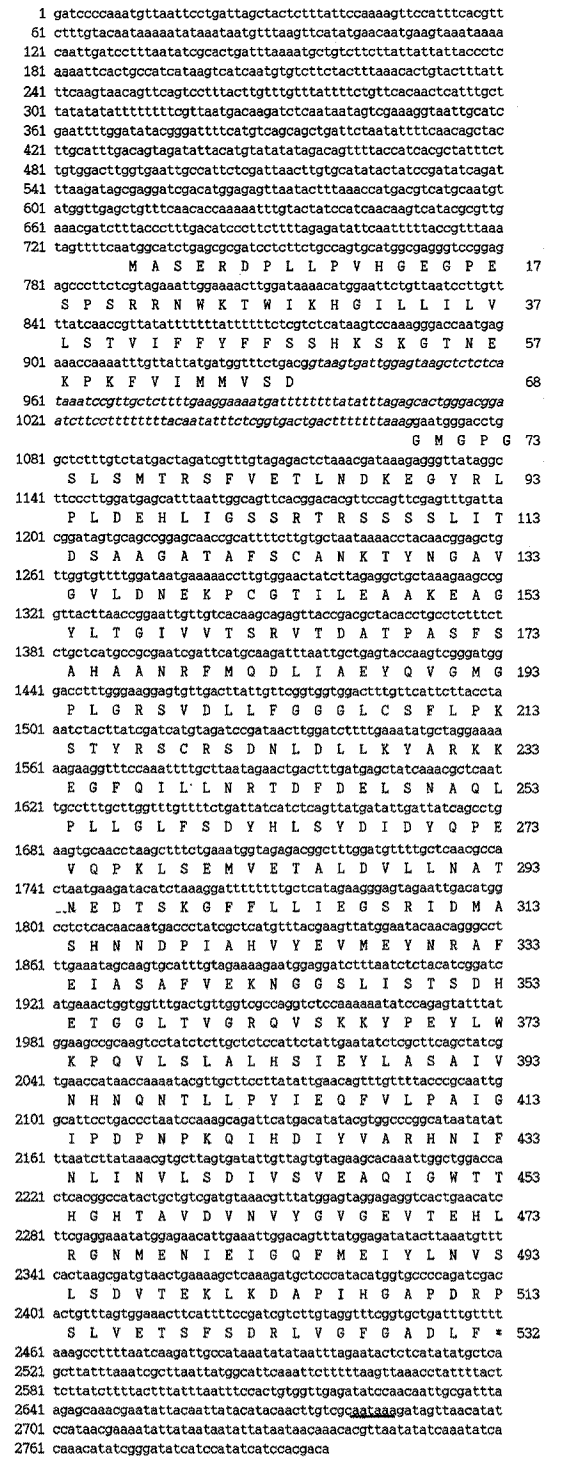

Fig. 2. The nucleotide sequence of the *Pst*I-*Bam*HI insert in the plasmid pSW20 that encodes alkaline phosphatase of *S. pombe*, and the deduced amino acid sequence of the enzyme. The intron sequence is represented in Italics. The number of nucleotides and deduced amino acids are marked in the left and right margins, respectively. An asterisk indicates the stop codon. The putative polyadenylation signal is underlined.

clearly demonstrates that the plausible AP gene is transcribed and functional.

Genomic DNA The two synthetic primers (5'-CCTGGATG GTTAGGGATCCCCAA ATGTTAAT-3'; primer 2, 5'-AGG TAATCGCGGCTGCAGTGGATGATAT-3') were used for PCR amplification by *Pyrobest*® DNA polymerase (TaKaRa

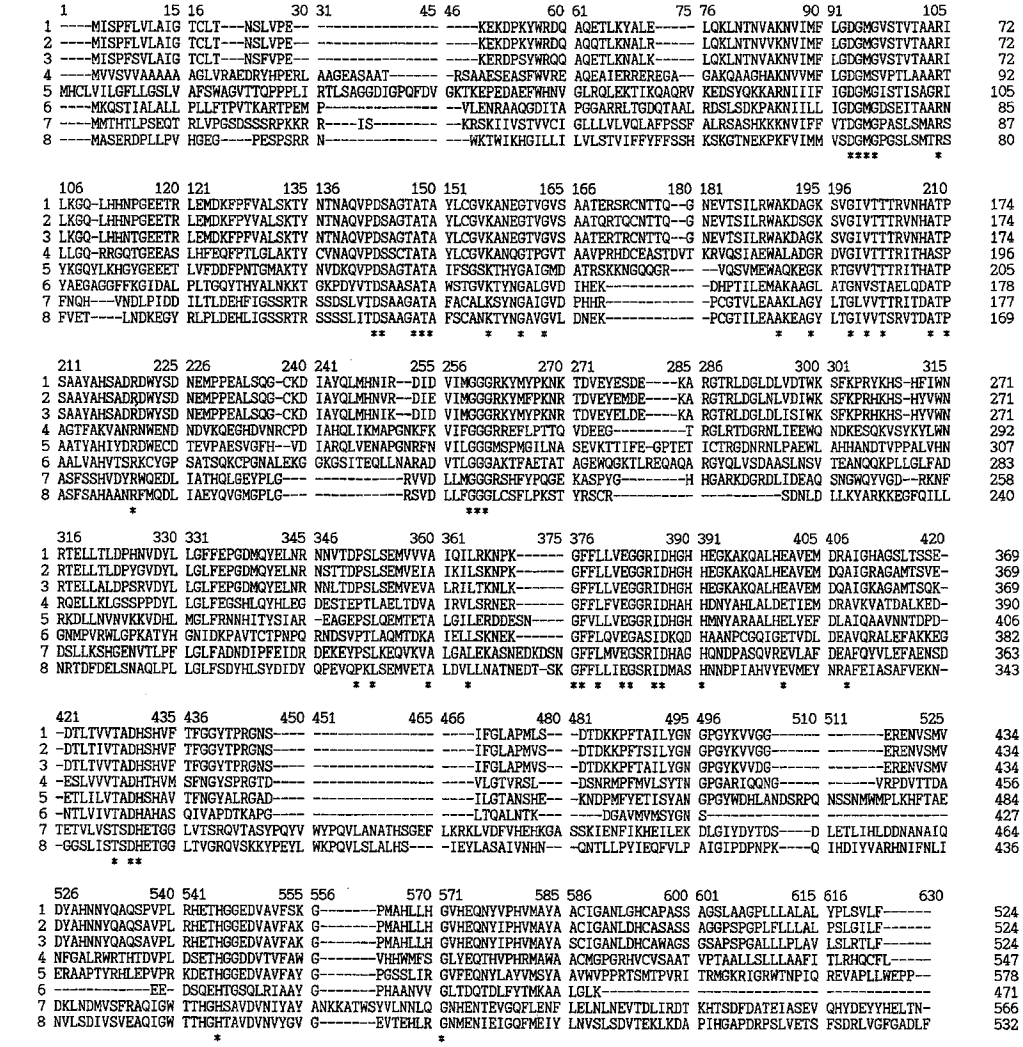

Fig. 3. Amino acid sequence alignment of *S. pombe* alkaline phosphatase with the most similar sequences identified in the GenBank database. Protein sequences deduced from alkaline phosphatase gene from *Homo sapiens* (sequence 1; Weiss *et al.*, 1986), *Felis catus* (sequence 2; Ghosh and Mullins, 1995), *Mus musculus* (sequence 3; Misumi *et al.*, 1988), *Bombyx mori* (sequence 4; Itoh *et al.*, 1991), *Drosophila melanogaster* (sequence 5; Yang *et al.*, 2000), *Escherichia coli* (sequence 6; Bradshaw *et al.*, 1981), *Saccharomyces cerevisiae* (sequence 7; Kaneko *et al.*, 1987) and *S. pombe* (sequence 8; this study, AF316541), are indicated by a standard single letter notation. Asterisks indicate the identical amino acids in the compared alkaline phosphatases.

Shuzo Co., Ltd., Japan). The amplified fragment contains *Pst*I and *Bam*HI restriction sites. The two primers were designed to amplify the AP coding region and 729 bp upstream sequence, which should contain sufficient region for transcriptional regulation. The amplified DNA product was identified and purified from an agarose gel electrophoresis, and then completely digested with *Pst*I and *Bam*HI. The digested PCR product was ligated into the *Pst*I/*Bam*HI site of the *E. coli*-yeast shuttle vector pRS315 to generate plasmid pSW20. Plasmid pSW20 contains a 2,800 bp insert. To determine the nucleotide sequence, two subclones were constructed using the unique *Hind*III site within the insert of plasmid pSW20. Plasmid pSW21 contains the *Pst*I-*Hind*III fragment, whereas plasmid pSW22 contains the *Hind*III-*Bam*HI fragment. Plasmid DNAs pSW21 and pSW22 were purified using the Spin Minipreps DNA Purification System (Core-Bio System, Ltd., Korea), and subjected to automated sequencing. The nucleotide sequence of the *S. pombe* AP gene was submitted to the GenBank under the accession number AF316541. The 2,797 bp sequence of the cloned AP gene is shown in Fig. 2. The coding region contains an intron. One putative polyadenylation signal is found at the downstream. The unique open-reading frame encodes a protein of 532 amino acids with a calculated mass of 58,666 daltons. The isoelectric point of the putative AP is 5.29. The computer analysis gave a sequence alignment of the *S. pombe* AP with other APs (Fig. 3). It is homologous with APs from *S. cerevisiae*, *Homo sapiens*, *Felis catus*, *Mus musculus*, *Bombyx mori*, *Drosophila melanogaster* and *Escherichia coli*. Homologous regions are more concentrated in the middle region. The amino acid

Table 1. Amino acid composition of alkaline phosphatase from *S. pombe*

class	amino acid	single letter	number	percentage
Positive	Arg	R	21	3.9%
	His	H	17	3.2%
	Lys	K	23	4.3%
Negative	Asp	D	30	5.6%
	Glu	E	34	6.4%
Polar	Asn	N	27	5.1%
	Cys	C	4	0.8%
	Gln	Q	13	2.4%
	Met	M	12	2.3%
	Ser	S	49	9.2%
	Thr	T	29	5.5%
Nonpolar	Ala	A	35	6.6%
	Gly	G	40	7.5%
	Ile	I	32	6.0%
	Leu	L	57	10.7%
	Pro	P	25	4.7%
	Val	V	37	7.0%
Aromatic	Phe	F	24	4.5%
	Trp	W	4	0.8%
	Tyr	Y	19	3.6%
	TOTAL		532	

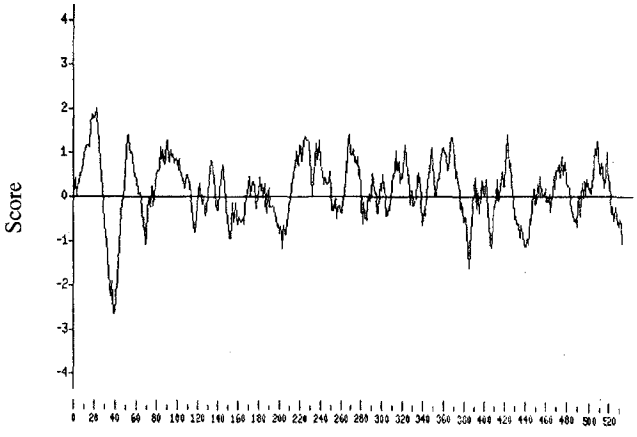

Fig. 4. Hydropathic profile of the amino acid sequence of alkaline phosphatase from *S. pombe*. It was analyzed using the Kyte-Doolittle method.

composition of the *S. pombe* AP is shown in Table 1. It is rich in leucine and serine. Its hydropathic profile is shown in Fig. 4. Hydrophobic regions are relatively prevalent, and hydrophobic and hydrophilic regions are alternatively positioned. When the phylogenic relationship was analyzed using PROTPARS, the *S. pombe* AP was very close to that of *S. cerevisiae* (Fig. 5). Their similarity has already been confirmed from sequence comparisons (Fig. 3).

Expression Plasmid pSW20 was transformed into *S. pombe*

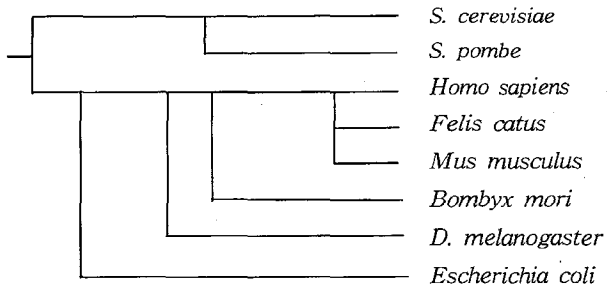

Fig. 5. Phylogeny of *S. pombe* alkaline phosphatase.

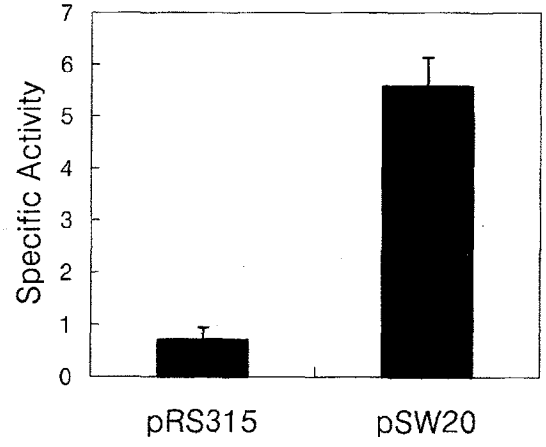

Fig. 6. Expression of the cloned alkaline phosphatase gene in *S. pombe* KP1 (h^+ *leu1-32 ura4-294*). Alkaline phosphatase activity was assayed as previously described (Harb *et al.*, 1991). Plasmid pSW20 harbors the cloned *S. pombe* alkaline phosphatase gene in the shuttle vector pRS315. The specific activity was represented by ΔA_{405}/min/mg protein.

KP1 (h^+ *leu1-32 ura4-294*). The yeast transformant was grown in minimal medium, and harvested at the exponential phase. Cell extracts were prepared from the yeast cultures, and used for an assay of AP activity. Yeast cells containing plasmid pSW20 showed much higher activity compared with the control cells (Fig. 6). This unambiguously demonstrates that the cloned *S. pombe* AP gene can produce functional AP. In the present communication, *S. pombe* AP cDNA and genomic DNA were isolated for the first time. The isolated genomic DNA was able to produce AP activity in *S. pombe*. However, its physiological role remains to be solved. Its purification and a regulation study would aid in elucidating the cellular function of the cloned AP. The fission yeast *S. pombe* would contain other kinds of APs. Further approaches will answer various questions on *S. pombe* AP.

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