Cloning and Sequence Analysis of cDNA for *Irpex lacteus* Aspartic Proteinase

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To find the primary sequence of *Irpex lacteus* aspartic proteinase (ILAP), a cDNA library of *I. lacteus* mRNA in pBR322 was constructed. A clone, which had an insert size of about 1.2 kilobase pairs, was found to contain the coding region of the mature enzyme. The deduced amino acid sequence showed that the enzyme consisted of 340 amino acid residues with a molecular weight of 35,000. Cysteine and methionine were not found in the enzyme and two putative *N*-glycosylation sites were indicated. The lack of S-S bridges in the molecule is a striking feature of the enzyme. The alignment of the sequence of the enzyme against other aspartic proteinases revealed homology around the active site aspartic acid residues.

In the selection of a rennet substitute, the ratio of milk-clotting activity to proteolytic activity (MCA/PA) is an important index, and a higher MCA/PA ratio is desirable. *Irpex lacteus* aspartic proteinase (ILAP) was most active at pH 3.0 toward protein substrates and is inhibited by pepstatin, diazoacetyl-D,L-norleucine methylester (DAN), and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP).¹⁾ It had almost the same value of MCA/PA as commercial microbial milk-clotting enzymes from *Mucor pusillus* and *M. miehei*. In actual cheese-making trials, ILAP could produce some kinds of cheeses of good quality including fiber-structured cheese,²⁾ Gouda cheese,³⁾ and Cheddar cheese.⁴⁾ All of the milk-clotting enzymes used commercially belong to the aspartic proteinase family and they are homologous when the primary structures are aligned and compared with other aspartic proteinases.⁵⁾ To get information on the structure-function relationship of ILAP, the elucidation of its primary structure is necessary. We described here the nucleotide sequence of ILAP cDNA. The ILAP sequence shows significant overall homology to the aspartic

proteinase family and strict conservation of essential residues around the two active-site aspartic acids.

Materials and Methods

DNA restriction and modification enzymes were from Takara Shuzo (Japan). Nitrocellulose filter was from Schleicher and Schuell. Oligo(dT)-cellulose (type III) was from Collaborative Research. ³²P-labelled nucleotides were purchased from Amersham. *Irpex lacteus* (KY2981) and its crude enzyme powder was kindly supplied by Kyowa Hakko Co., Ltd.

A medium containing 4% sucrose, 3% distillers solubles, 0.3% yeast extract, 0.5% KH₂PO₄, and 0.05% MgSO₄·7H₂O, pH 4.0, was used for seed culture and a medium containing 6% sucrose, 3% distillers solubles, 0.6% yeast powder, 0.6% NH₄H₂PO₄, 0.5% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.1% lactic acid, 1 ppm calcium pantothenate, 10 ppm choline chloride, and 0.2% Nonion E-230, pH 3.8, was used for enzyme production.

Purification of ILAP. The purification procedures were done at 4°C unless otherwise indicated. Crude enzyme powder was dissolved in ice-cold 0.01 M sodium acetate, pH 5.5, and ILAP was partially purified by affinity chromatography as described previously.¹⁾ Fractions rich in ILAP were dialyzed against 0.02 M sodium acetate, pH 4.6, and were put on a column of DEAE-Sepharose fast flow (1.5 × 30 cm) equilibrated with the same buffer. ILAP

was eluted from the column with a pH gradient from the buffer to 0.1 M acetic acid. Main fraction was collected and put on a RP-HPLC column (C_8 , Altex, 4×250 mm) equilibrated with 25 mM triethylamine-phosphate, pH 6.0. ILAP was eluted as a single peak with a linear acetonitrile gradient from 0% to 90% and used as purified ILAP. This preparation was judged homogeneous by SDS-PAGE⁶⁾ and pH 4.3 PAGE⁷⁾ (data not shown).

Amino acid analysis. For determination of the amino acid composition, purified ILAP was hydrolyzed in 6 N HCl at 110°C for 24, 48, and 72 hr, and the hydrolyzate was analyzed with a Dionex amino acid analyzer model D-5. Tryptophane was measured with *p*-toluenesulfonic acid by the method of Penke.⁸⁾

Preparation of *I. lacteus* mRNA. An agar block (5×5 mm) containing *I. lacteus* hyphae from a slant culture was inoculated into 100 ml of seed medium in a 500-ml Sakaguchi flask and cultured at 30°C for 3 days with shaking. Then 5 ml of this seed medium was inoculated into 100 ml of enzyme production medium in a 500-ml Sakaguchi flask and cultured at 30°C for 5 days. The mycelia were collected by filtration on No. 2 filter paper, washed with H_2O , frozen in liquid nitrogen and stored at -70°C until use.

A mixture of 80 ml of extraction buffer (0.1 M Tris-HCl, 10 mM EDTA, 1% SDS, pH 9.0), 40 ml of phenol saturated with the extraction buffer, and 40 ml chloroform was added to 20 g of frozen mycelium, and homogenized at 15,000 rpm for 3 min with a homogenizer (Ace, Japan). After centrifugation ($15,000 \times g$, 20 min, 2°C) the aqueous phase (92 ml) was recovered. RNA was selectively precipitated by making the aqueous phase 2 M in LiCl and standing for more than 1 hr at 2°C. The precipitate was collected by centrifugation ($15,000 \times g$, 15 min, 2°C), washed three times with 2 M LiCl and twice with 70% (v/v) ethanol at -20°C. From the total RNA obtained (36 mg), samples (14 mg) were put on an oligo(dT)-cellulose column and chromatographed. A messenger RNA fraction (375 μ g) was obtained and the recovery was estimated to be 2.7%.

cDNA synthesis and screening of the cDNA library. Oligo(dT)-primer was elongated by reverse transcription of poly(A^+)RNA, and double-stranded cDNA was synthesized as described by Gubler and Hoffman.⁹⁾ The resulting double-stranded cDNA was treated with T_4 DNA polymerase to remove the remaining 3'-end overhangs from the first-strand cDNA and inserted into the *Pst* I site of pBR322 by the (dG)-(dC) homopolymer extension method.¹⁰⁾ *E. coli* DH-1 cells were transformed with the recombinant plasmids as described by Hanahan.¹¹⁾ The yield of transformants was about 10,000. The cDNA library was screened for ILAP cDNA sequences by colony hybridization with ³²P-labeled oligonucleotide probe.

Prehybridization and hybridization were done at

36°C in a solution containing $6 \times$ SSC, 0.5% SDS, $5 \times$ Denhardt's solution, and 100 μ g denatured *E. coli* DNA/ml. ($1 \times$ SSC is 0.15 M NaCl/0.15 M sodium citrate buffer, pH 7.0; $1 \times$ Denhardt's solution contains 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone). For hybridization, a 5'-labeled oligonucleotide probe was added to the solution. Filters were washed for 5 min in $6 \times$ SSC/0.1% SDS at 0°C, followed by three successive 20 min washings in 3 M Me₄NCl at 50°C as described by Wood.¹²⁾ Positive colonies were detected by autoradiography at -70°C with Fuji RX film and an intensifying screen.

Northern blot analysis. RNA-blot analysis of *I. lacteus* poly(A^+)RNA was done. RNA was denatured with glyoxal and dimethylsulfoxide and resolved by 1% agarose gel electrophoresis in 12 mM sodium acetate-0.2 mM EDTA, pH 7.0. The RNA was transferred overnight to a nylon filter (Gene Screen Plus, Du Pont) with $10 \times$ SSC. The filter was dried at room temperature. RNA blots were prehybridized at 60°C for 15 min in a hybridization buffer (12.5% dextran sulfate, 1 M NaCl, 1% SDS, and 100 μ g denatured *E. coli* DNA/ml). Hybridization was done overnight at 60°C with the nick-translated cDNA as a probe. The filter was washed twice at room temperature with $2 \times$ SSC and once at 60°C with $2 \times$ SSC/1% SDS and once at room temperature with $0.1 \times$ SSC for 1 hr before drying and autoradiography.

Results and Discussion

N-Terminal amino acid sequence of ILAP

The NH₂-terminal sequence of ILAP was found by Edman degradation to be A-A-G-S-V-P-A-T-N-Q-L-V-D-Y-V-V-N-V-G-V-G-S-P-A- and used to design oligonucleotide probe for finding ILAP cDNA from the library.

Isolation of a cDNA clone for ILAP

A cDNA library in pBR322 was constructed and screened with a oligonucleotide probe corresponding to the amino acids from 13 to 18 of ILAP sequence (Fig. 1). From the 10,000 colonies screened, 300 positive clones were obtained. Recombinant plasmids from the selected colonies were prepared, digested with *Pst*I, electrophoresed in agarose gel, and then transferred to nitrocellulose filters. Southern blot analysis showed that a clone, pILAP 4108, had an insert of about 1.2 kilobase pairs that hybridized with the probe. The other clones

had positive inserts of less than 0.8 kilobase pairs and they were insufficient in length to encode mature ILAP. Thus, the sequence of this clone with the longest insert (pILAP 4108) was analyzed.

Nucleotide sequence analysis

The insert from the clone was analyzed with a series of restriction enzymes. Figure 2 shows the restriction map and sequencing strategy. The nucleotide sequence of the insert is shown in Fig. 3 and the amino acid sequence of 340 residues of ILAP is deduced from the cDNA sequence. The NH₂-terminal sequence deduced from the Edman degradation of the

protein was found to start at residue 1 in Fig. 3 (starting at nucleotide 3). The COOH-terminal position was found at N 340 (nucleotides 1020~1022) which immediately precedes a termination codon, TAG. The amino acid content from amino acid analysis agrees well with that calculated from the sequence data (Table I). Therefore, from these results, it is reasonable to conclude that this cDNA encodes ILAP.

The sequence analysis shows that the clone pILAP 4108 does not contain the entire pro-

Fig. 1. Oligonucleotide Probe Used for Identifying ILAP cDNA.

Fig. 2. Restriction Map and Sequencing Strategy of ILAP cDNA.

GT	GCC	GCT	GCG	AGC	GTC	CCC	GCC	ACC	AAC	CAG	CTC	GTA	GAC	TAC	GTT	GTT	AAC	GTT	GCG	GTC	GCG	TCC	CCC	GCC	74
A	A	G	S	V	P	A	T	N	Q	L	V	D	Y	V	V	N	V	G	V	G	S	P	A		24
ACC	ACT	TAC	TCT	CTC	TTG	GTC	GAT	ACC	GGT	AGC	TGG	AAC	ACC	TGG	CTC	GGT	GCT	GAC	AAG	TCT	TAT	GTC	AAG	ACT	149
T	T	Y	S	L	L	V	D	T	G	S	N	T	W	L	G	A	D	K	S	Y	V	K	T		49
TCC	ACC	TGG	TCT	GCG	ACC	AGC	GAC	AAA	GTG	TCC	GTC	ACC	TAC	GGA	TCC	GCC	TGG	TTC	TCT	GGC	ACT	GAA	TAC	ACT	224
S	T	S	S	A	T	S	D	K	V	S	V	T	Y	G	S	G	S	F	S	G	T	E	Y	T	74
GAC	ACC	GTC	ACT	CTC	GGA	AGC	TTG	AGC	ATC	CCC	AAG	CAA	TCC	ATC	GGT	GTT	GCC	TCC	GAT	AGC	GGA	TTG	GAT		299
D	T	V	T	L	G	S	L	T	I	P	K	Q	S	I	G	V	A	S	R	D	S	G	F	D	99
GGT	GTT	GAT	GGT	ATC	CTC	GGT	GTC	GCG	CCT	GTC	GAC	CTT	ACT	GTC	GGT	ACC	CTC	AGC	CCC	CAC	ACC	TCT	ACC	TCC	374
G	V	D	G	I	L	G	V	G	P	V	D	L	T	V	G	T	V	G	T	D	S	S	T		124
ATC	CCC	ACT	GTC	ACC	GAC	AAC	CTC	TTC	AGC	GAG	ACC	ATC	CCC	ACC	AAC	TTG	CTC	GCG	GTC	TCC	TTC	GAG	CCC		449
I	P	T	V	T	D	N	L	F	S	Q	G	T	I	P	T	N	L	L	A	V	S	F	E	P	149
ACC	ACC	TGC	GAG	TGG	AGC	ACC	AAC	GCG	GAG	TTG	ACT	TTC	GGT	GCT	ACT	GAC	TCC	AGC	AAG	TAC	ACT	GGT	TCC	ATT	524
T	T	S	E	S	S	T	N	G	E	L	T	F	G	A	T	D	S	S	K	Y	T	G	S	I	174
ACT	TAC	ACT	CCC	ATC	AGS	AGC	ACC	TCC	CCC	GCG	TCT	GCG	TAC	TGG	GGT	ATT	AAC	CAG	AGS	ATT	CGC	TAC	GCC	TCT	599
T	Y	T	P	I	T	S	T	S	P	A	S	A	Y	W	G	I	N	Q	T	I	R	Y	G	S	199
TCC	ACC	TCC	ATC	CTC	TCC	TCC	ACC	GCC	GGT	ATC	GTC	GAC	ACT	GCC	ACC	CTC	AGS	CTC	ATT	GCT	TCT	GAT	GCG		674
S	T	S	I	L	S	S	T	A	G	I	V	D	T	G	T	T	L	T	L	I	A	S	D	A	224
TTC	GCC	AAG	TAC	AAG	GCT	ACT	GCT	GCT	GTT	GCT	GAC	AAC	AAC	ACT	GGT	CTC	TTG	GCG	TTG	ACC	GCT	ACC	GCT	CAG	749
F	A	K	Y	K	K	A	T	G	A	V	A	D	N	N	T	G	L	L	R	L	T	T	A	Q	249
TAC	GCC	AAC	CTC	CAG	AGC	TTG	TTT	ACC	ATT	GGT	GCG	CAG	ACT	TTC	GAC	CTG	ACC	GCC	AAC	GCT	CAG	ATC	TGG		824
Y	A	N	L	Q	S	L	T	I	G	G	I	G	Q	T	F	E	L	A	N	A	Q	I	W		274
CCC	GCT	AAC	CTG	AAC	AGC	GCT	ATT	GGT	GCC	AGC	GCC	AGC	AGT	GTT	TAC	CTC	ATC	GTT	GGT	GAC	CTC	GCC	TCC	GAC	899
P	R	N	L	N	T	A	I	G	G	S	A	S	S	V	Y	L	I	V	G	D	L	G	S	D	299
AGC	GCG	GAG	GGT	CTC	GAC	TTT	ATC	AAC	GGT	TTG	ACC	TTC	CTC	GAG	GGT	TTC	TAC	TCT	GTC	TAC	GAC	ACC	AGC	AGC	974
S	G	E	G	L	D	F	I	N	G	L	T	F	L	E	R	F	Y	S	V	Y	D	T	T	N	324
AAG	AGG	CTT	GGT	TTG	GCC	ACC	ACC	TCC	TTC	ACC	ACC	GCC	ACC	TCC	AAC	TAG	ACGTTGGCTGACGAAACGGTGGCAAGCTGGAA							1056	
K	R	L	G	L	A	T	T	S	T	T	A	T	S	N	***										340
AAGACAGTGAATGTACAAATGGTGGGATCATGCACTGTTTATGGAAATGTATGCTCTGTGAACGGAAATGGAAATTTGGAAATGGATGTTGTTATGTTT																									1155
TTCTGCTAT																									1164

Fig. 3. Nucleotide Sequence and Deduced Amino Acid Sequence of ILAP cDNA.

N*, putative glycosylation site; D⁺, active site aspartyl residue; ***, termination codon. The underline indicates the amino acid sequence which is coincident with the N-terminal amino acid sequence found by Edman degradation of the purified ILAP.

Table 1. AMINO ACID COMPOSITION AND CODON USAGE OF *I. lacteus* MILK-CLOTTING ENZYME

Amino acid	No. of residues*		No. of codons found
	A	B	
D		18	13 GAC 5GAT
	35		
N		16	16 AAC
T	53	55	32 ACC 16 ACT 7 ACG
S	46	47	19 TCC 9 TCT 4 TCG 14 AGC 1 AGT
E		7	6 GAG 1 GAA
	17		
Q		8	7 CAG 1 CAA
P	11	11	10 CCC 1 CCT
G	37	36	19 GGT 14 GGC 3 GGA
A	27	26	11 GCC 10 GCT 5 GCG
C	0	0	
M	0	0	
V	24	23	13 GTC 8 GTT 1 GTA 1 GTG
I	17	17	11 ATC 6 ATT
L	31	30	17 CTC 2 CTT 2 CTC 9 TTG
Y	14	14	13 TAC 1 TAT
F	12	13	12 TTC 1 TTT
K	8	9	8 AAG 1 AAA
H	1	1	1 CAC
R	5	6	3 CGT 2 CGC 1 AGG
W	2	3	3 TGG
Total	340	340	

* A, amino acid analysis; B, nucleotide sequence.

tein coding region. In addition, this clone neither contains the polyadenylation signal normally found in eukaryotic mRNA nor a poly(A) tail.

It is well known that aspartic proteinases have bilobal structures with a well-defined active site cleft. The two lobes comprise the N-terminal and C-terminal regions of these enzymes, and are topologically similar, indicating a possible mechanism in the evolution of aspartic proteinases by gene duplication and fusion.¹³⁾ The amino acid sequence of ILAP is compared with those of other aspartic pro-

teinases, including penicillopepsin, porcine pepsin, and some milk-clotting enzymes, in Fig. 4. The aligned structures have identical residues in 20 positions. Most of them (15 residues) are in the N-terminal region (residues 1~170) of the structure and there are 5 residues in the C-terminal region, but the significance of this difference is not obvious. The sequence identity of ILAP with microbial milk-clotting enzymes such as endothiasepsin, *Mucor pusillus* and *M. miehei* aspartic proteinases were 26.2%, 22.6% and 22.4%, respectively. The identity of ILAP with penicil-

Fig. 4. Alignment of ILAP with Other Aspartic Proteinases.

The numbering of residues is based on the penicillopepsin sequence. ILAP, *Irpex lacteus* aspartic proteinase; PEP, penicillopepsin¹⁴⁾; RHP, rhizopuspepsin¹⁵⁾; RNAP, *Rhizopus niveus* aspartic proteinase¹⁶⁾; ENP, endothiasepsin⁵⁾; MPAP, *Mucor pusillus* aspartic proteinase¹⁷⁾; MMAP, *Mucor miehei* aspartic proteinase¹⁸⁾; YPA, yeast proteinase A¹⁹⁾; POP, porcine pepsin^{20~22)}; MOP, monkey pepsin²³⁾; HUP, human pepsin²⁴⁾; CHP, chicken pepsin²⁵⁾; POCD, porcine cathepsin D²⁶⁾; HUCD, human cathepsin D²⁷⁾; MOG, monkey gastricsin²⁸⁾; BOC, bovine chymosin^{29~31)}; HUR, human renin^{32,33)}; MOR, mouse renin^{34,35)}; RAR, rat renin.³⁶⁾

ILAP 1 10 20 30 40 50 60 70 80
PEP AAGSVCATP--N-QLVDVNVGVCSPATTSYLSLVDGSSSNMLGA-----DKSVVK--TST-S-SATSD-KVSVTYGSGS-FSCTE
RHP AASGVAINTPA-NDREYVTPWTIG--GTTLANPDTGCSADLWFSSTELPA-----SQQSQSVNVP--SAT-GKELSGI---TMSISYGDGSSASGVN
RNP AGCVTVMET--DYQNDVEYGVQVITGCGKFNLDFTGSSDLWASTLCTN-----CGRS-QTKYDP--KGS-S-TYQADGRWISYGDGSSASGIL
ENP AASGVNMP--DYADNVEYGVQVITGCGKILKLDFTGSSDLWFSSTELPA-----SEVDQVITPTPSKCTIL-LISGA---TMSISYGDGSSSSGD
MPAP STGSAVTPFD--S-LDAVYFVQVITGCTAQLADLWFSSTELPA-----SEVDQVITPTPSKCTIL-LISGA---TMSISYGDGSSSSGD
MPAP AEGDSVTPFGL--YDFLEELAPVLSIGTPQDFLLFTDGTSSDLWFSKGCNS-EGC--VGRGFPDPSSSSTFK--ETDY--NLNITVGTGGAN-GIY
YFA GAHNVPLT--NLYNAQYVGTITLGTGPQNFVLFTDGTSSDLWFSVCSLL--AC--FLSKNDHEASSYK--ANGT--EFAIQGTGGL-GYT
POP IGDHFE--NLYLDFEFTGILGTGAQDFVLFDTGSSNLWSPSVCSLL--AC--SDHNKPNPDSDSTFE--ATGQ--ELSTYTGTCGM-TGIL
MPAP LDEHFE--NLYLDFEFTGILGTGAQDFVLFDTGSSNLWSPSVCSLL--AC--INLNPNDPDSDSTY--STSQ--TLSITYTGTCGM-TGIL
HUP VDEHFE--NLYLDFEFTGILGTGAQDFVLFDTGSSNLWSPSVCSLL--AC--INLNPNDPDSDSTY--STSQ--TLSITYTGTCGM-TGIL
POCD TATESYFEMT--NLYMDASYGTLSIGTPQDFVLFDTGSSNLWSPSVYKSS--AC--SNEKRFDPSSKSTYV--STNE--TVSYTGTCGM-SGIL
HUR GPTFEVTK--NLYMDASYGTLSIGTPQDFVLFDTGSSNLWSPSHQKLLDLC--WIHKYNSGKSSSTYV--KNGT--TFALHYGSGIL-SGYL
HUCD GPTFEVTK--NLYMDASYGTLSIGTPQDFVLFDTGSSNLWSPSHQKLLDLC--WIHKYNSGKSSSTYV--KNGT--TFALHYGSGIL-SGYL
BOG SVSYFEMA--NLYMDASYGTLSIGTPQDFVLFDTGSSNLWSPSVYQSO--AC--TSHSRPNPSSSTYV--TNQK--TFSLYQSGIL-TGFF
MPAP GEVASVPLT--NLYDSYVFGKILGTGPQDFVLFDTGSSDLWFSVSIYCS--NAC--KNHRFRDPKSSSTYV--NQK--PLSIYTGTCGM-QGIL
HUR LTLNGTSSVLLT--NLYMDTYGTLSIGTPQDFVLFDTGSSNLWSPSSKSYLYAC--VYHLKFDASDSSSYK--HNGT--ELTYTGTCGM-SGIL
MOR SSLLDILSPFVLT--NLYNSQYGEILGTGPQDFVLFDTGSSNLWSPSKRSRLYAC--GHLSYSDSSSSSY--ENGT--DFTHYGSGRKV-GFL
RAR SSFTNVISPVLT--NLYDTQYGTLSIGTPQDFVLFDTGSSNLWSPSKOCPILYAC--EHLNLYDSSSSSYM--ENGT--EFTHYGSGRKV-GFL
COMMON Y G DTGS W Y G G

ILAP 90 100 110 120 130 140 150 160
PEP YTDVFTL--GSLTIKQSGIVASRGS-FD--GV--DGLIGVGVVLVITGLSPHTSITPTVTDNLPSQGTITPNLAVSFPTTS
RHP FTDVFTV--GVTHAQVAVQAQVISAQFO--QDTNN--DGLGLAFS--SNTLVPSQSGTTFDVKSSLAQ--PLFVAKLHQPOK
RNP AKDNNVL--GGLLIGKQITELAKRESSAFA--NGPNDLGLGFDITTVR--GKVTMDNLISQGLSRIPGVYLGKSN
MPAP ATANNL--GGLLIGKQITELAKRESSAFA--TVDLGLGLGFDITTVR--GKVTMDNLISQGLSRIPGVYLGKSN
ENP YTDVFTV--GCLTVTQAVESAKVSSSPT--EDSTT--DGLGLAFSTLNTVS--PTQKQTFEDNAK--ASLDSPLVITADLG
MPAP FRTSITV--GCAVTKQVILAVDMSVPTAEGSPNADFLDGLFGAAYPONTAMEA--EYGVTDNTVHVNLYQGLJSSPVSVN--TN
MPAP FEDSLAI--GDTIVTKQLAVDMSVPTAEGSPNADFLDGLFGAAYPONTAMEA--EYGVTDNTVHVNLYQGLJSSPVSVN--TN
POP SLDITLPT--GDLTIKQFAEATSEPLIT--AFKGFGLDGLGDTISVK--VP--PPYNAQDQLDLKELFVYVYLGJTSK
HUP GYDITVQV--GGLSDINQIFGLSETPEPSGL--YAAPFDGILGLAYPSISAGS--ATP--VFDNLWDQGLVSQLDPSVYLS--SN
MPAP GYDITVQV--GGLSDINQIFGLSETPEPSGL--YAAPFDGILGLAYPSISAGS--ATP--VFDNLWDQGLVSQLDPSVYLS--AD
HUP GYDITVQV--GGLSDINQIFGLSETPEPSGL--YAAPFDGILGLAYPSISAGS--ATP--VFDNLWDQGLVSQLDPSVYLS--AD
HUR GYDITVAV--SSLVDQIFGLSETPEPSGF--YVNPFDGILGLAFPSISAGS--ATP--VFDNMHMQGLVSQLDPSVYLS--KD
POCD SQTIVSPVSN--VGGLKVERQVTEATKQPLGLT--IAKFDGILGLAYPRISVNN--VVP--VFDNLWDQGLVSQLDPSVYLS--RD
HUCD SQTIVSPVSSASASALGVKVERQVTEATKQPLGLT--IAKFDGILGLAYPRISVNN--VLP--VFDNLWDQGLVSQLDPSVYLS--RD
BOG GYDITVAV--QSLVQVQFGLSENEPGFN--VYAFDGLGLAYPTLSDVG--ATT--AMQGVQEGALTSPIQPSVYLS--DQ
MPAP GYDITVSN--IVDIQ--QIVGLSGQVDF--TAAFDGILGLAYPSLASEY--SIP--VFDNMHNRHLVSQLDPSVYMDRIG
HUR SODLITV--GGTIVT--QMFGEVTRPALP--MLAETDGLVGLMPEIQAR--VTP--IFDNIISQGLVQKDFVSFYNRDSE
MOR SODSVTV--GGTIVT--QTFGEVTEPLIPF--MLAETDGLVGLMPEIAQVG--VTP--VDTHLSQGLVQKDFVSFYNYNRG--
RAR SODSVTV--GGTIVT--QTFGEVTEPLIPF--MLAETDGLVGLMPEIAQVG--VTP--VDTHLSQGLVQKDFVSFYNYNRG--
COMMON Q DG G

ILAP 170 180 190 200 210 220 230 240
PEP ESSINGELT-FGATDSSKYTCSLYTPTISTSPASAYWQINQITRGSSSTSLSS--STAGI--VDTCITTLTIASDAFAKYKKA-TGVAADNNTGLRL
RHP --GYVD-FGFIIDSSKYTCSLYTGVINS--QGFWSFND-SYTAGSDGSG--GFSGI--ADTGCTITLLLDSDSVYQVYSGAAGDSNAGGTVF
RNP GGG--GEYV-FGGSYTFKFGSLITVFDINS--RGMGITVTRAVTGSTVAS--SFDGI--LDTGTTLLLPNNVAASVARY-GASDN-GDGYTIT
RNP GGG--GEYV-FGGSYTFKFGSLITVFDINS--EGFWGVZKSTKIGTSTVAS--SFDAI--LDTGTTLLLPNNVAASVARY-GASDN-GDGYSTI
ENP YHAP-GTYN-FGFIITDITATYSITVAVSTQ--GEWESVT-GVAGSGTKST--SLDGI--ADTGTTLLLYLATVSPAYWAKVQSSSSSGVGYV
MPAP GGG--QGVV-FGGMNNTLIGDITVTDWIKSRGYSVDFADPVT-GVKIDSGSDVAD-SFDQAQFATITDGTNFIIPSSAGAKVKAALPDATESQOQ-YTV
MPAP STT--GEVV-FGGMNNTLIGDITVTDWIKSRGYSVDFADPVT-GITVDGSGAV-RFSRQAPATITDGTNFIIPSSAGAKVKAALPDATESQOQ-WVV
POP TEN--GREATFTDKSFKGDTLTVPR--KAYMEVFE-GILGDEAYE--LESHGAA--IDTGTSLITPSGLAEMI-NAETGA-KMGATGYTL
RNP DGS--GSVVLFGIDSSSYTGSNLWPNVSV--EGYQWITD-SITMDGTEA-CSSGQOAT--VDGTGSLITGTPSAIAINQSDIGA-SENSDGMV
MPAP AQP--GSVVLFGIDSSSYTGSNLWPNVSV--EGYQWITD-SITMDGTEA-CSSGQOAT--VDGTGSLITGTPSAIAINQSDIGA-SENSDGMV
HUP DGS--GSVVLFGIDSSSYTGSNLWPNVSV--EGYQWITD-SITMDGTEA-CSSGQOAT--VDGTGSLITGTPSAIAINQSDIGA-SENSDGMV
MPAP GET--GSVVLFGIDSSSYTGSNLWPNVSV--ETVQWITD-RVTNGKRYA-CFQOQAT--VDGTGSLITGTPSAIAINQSDIGA-SENSDGMV
POCD AQP--GGEMLIGDSSSYTGSNLWPNVSV--KAYWHLND-QUEVASGLIT--CKGCEAI--VDGTGSLITGTPSAIAINQSDIGA-SENSDGMV
HUCD AQP--GGEMLIGDSSSYTGSNLWPNVSV--KAYWHLND-QUEVASGLIT--CKGCEAI--VDGTGSLITGTPSAIAINQSDIGA-SENSDGMV
BOG QCS--SGGAVFGGVDSSLYTGQIYVAPVTV--ELWYQIGIE-EFLIGQASGAGGEGQAT--VDGTGSLITGTPSAIAINQSDIGA-SENSDGMV
MOR QCS--ESMLTGAIDPSYTGSLWPNVTV--QYVQVYFVD-SVTISGVAWA-CEGQOAT--VDGTGSLITGTPSAIAINQSDIGA-SENSDGMV
HUR NQSGLGQIYVFGGVDSSLYTGQIYVAPVTV--TGWQYQIMK-GVSGVSSSTLL-CEGQOAT--VDGTGSLITGTPSAIAINQSDIGA-SENSDGMV
HUR NQSGLGQIYVFGGVDSSLYTGQIYVAPVTV--TGWQYQIMK-GVSGVSSSTLL-CEGQOAT--VDGTGSLITGTPSAIAINQSDIGA-SENSDGMV
RAR NQSGLGQIYVFGGVDSSLYTGQIYVAPVTV--TGWQYQIMK-GVSGVSSSTLL-CEGQOAT--VDGTGSLITGTPSAIAINQSDIGA-SENSDGMV
COMMON G A G DTG

ILAP 250 260 270 280 290 300 310 320
PEP TTAQYANQLSFFTIQGQITELTANQALWPRNLNIAAGSSAVSVLIVDGLG--SDSGEGTIFINGTLFLRYFSVYDITNRK-LGLATTSFITTATSN
RHP DCS--SSIPDQSVSISGYTAVPGLSINGVSGNGST--CLAGTG--SNSGIGF-SINGDLFQSGVYVVDSDGQO-LFQAPOA
RNP SCDT-SRKPLPVSINGASQVPSDLVFEK--YQOQ--CIAGF--GYNDFDAIIGDITLGNVYVNGVQVPE-VQIAVPAQ
RNP CDT-SKQPLVFTSINGSTPEVSDSLIFEK--DNK--CIAGF--AAG-DGAILLGDVFLNNYVNGVQVPE-VQIAVPAQ
ENP PCSA--TSLTIGDQSGARVILPGYIDRPGISTGSS--CPFGTG--SSAGIGI-NIFGDAVLAARVFNNGATITPTTGLFAP
MPAP PCKSY--QDSKTFISLQDGSSTSDIVDVSPIKMLPVDKSGEOTGFI--VLDPGQGVITLNGLLAFRVVYDGRNK-IGFAPLASGYNN
MPAP PCASY--QNKSTISVNMKSGSSSDITELVSPVSKMLPVDQSNETOQFI--LIDPGQGVITLNGLLAFRVVYDGRNK-IGFAPLASGYNN
YFA DONTNRNLDLIFNNGNYTITGPYDITVSGS--CISATFP--MDPEFVPLAIVGDADFLKRSYSYDGLN-IGFALAI
POP SCSSISLDPDITVTDGQVITPSFAYILQDGS--CISGFG--MDVPTSSGELIIGDVIROYVYVDFRANK-VGLAFAV
MPAP SCSSISLDPDITVTDGQVITPSFAYILQDGS--CISGFG--MDVPTSSGELIIGDVIROYVYVDFRANK-VGLAFAV
HUP SCSSISLDPDITVTDGQVITPSFAYILQDGS--CISGFG--MDVPTSSGELIIGDVIROYVYVDFRANK-VGLAFAV
HUR SCSSISLDPDITVTDGQVITPSFAYILQDGS--CISGFG--MDVPTSSGELIIGDVIROYVYVDFRANK-VGLAFAV
POCD PCKSVPLSDPITVLDGKQYKSSNYTKVQSQGTLT--CLSGFM--MDLPPSGPLIIGDVIROYVYVDFRANK-VGLAFAV
HUCD PCKSVPLSDPITVLDGKQYKSSNYTKVQSQGTLT--CLSGFM--MDLPPSGPLIIGDVIROYVYVDFRANK-VGLAFAV
BOG NCNSIQNLPTLIT

lopesin was 28.2%, 28.5% with rhizopuspepsin, and 27.1% with porcine pepsin. The amino acid residues in ILAP identical to those in penicillopepsin, rhizopuspepsin or porcine pepsin are distributed through out the molecules, which suggests that the tertiary structure of ILAP should be similar to those of penicillopepsin, rhizopuspepsin, and porcine pepsin.

Compared with the sequence of penicillopepsin, there is a deletion of 10 residues (around the 50th residue) and two insertions of 5 residues (around the 150th and 165th residues) in the N-terminal region and one insertion of 10 residues (between the 280th and 290th residues) in the C-terminal region of ILAP. The regions which correspond to the residues 40~60 and 270~290 in penicillopepsin are appreciably variable among aspartyl proteinases. These regions are on the surface of the penicillopepsin molecule away from the active site cleft and are thought to be unimportant for activity.^{13,14)} Accordingly, it can be inferred that the large deletions and insertions in ILAP occur in these variable regions.

The inactivation of ILAP by active-site directed reagents of pepsin such as DAN and EPNP has implied that the catalytic apparatus of ILAP is similar to those of aspartic proteinases.¹⁾ Comparison of the sequence of ILAP with those of other aspartic proteinases suggests that the regions 33~36 and 213~215 in penicillopepsin are highly conserved among those enzymes and D 32 and D 212 in ILAP are the active site aspartic acid residues. Y 75 (Y 63 in the ILAP sequence) is thought to be the residue that participates in the catalytic mechanism,³⁷⁾ and the residues from 75~79 are also well conserved among aspartic proteinases including ILAP.

The molecular weight of ILAP calculated from the predicted sequence was 35,000 and two putative *N*-glycosylation sites, N-X-T, were found at 192 and 238. This molecular weight is apparently smaller than that found by SDS-PAGE (39,000). The difference seems to be attributed to its sugar chain which can be

attached to Asn 192 and/or Asn 238. ILAP seems to have one or two sugar chains, because ILAP shows an affinity towards various kinds of lectins including concanavalin A, wheat germ agglutinin, and *Ricinus communis* agglutinin (data not shown). From the alignment shown in Fig. 4, it seems that the two putative glycosylation sites of ILAP can be located on the surface of the molecule, because the regions near 190 and 240 in penicillopepsin are exposed to the atmosphere.¹³⁾

One striking feature of the amino acid composition of ILAP is the high content of T and S, like endothiapepsin. Contents of T and S of endothiapepsin and ILAP were calculated to be 29% and 30%, respectively, and for both residues they are double the average values for other proteins.⁵⁾

The other striking feature of ILAP is the absence of cysteine and methionine in its primary structure. This suggests that ILAP is the first aspartic proteinase which contains no disulfide bridge in the molecule. It is interesting to note that the number of disulfide bonds is three in vertebrate aspartic proteinase, two in rhizopuspepsin, *Mucor pusillus* as-

Fig. 5. Northern Blot Analysis of *I. lacteus* Poly (A⁺) RNA.

5 μ g of mRNA were separated on a 1% agarose gel, blotted to nylon filter, and hybridized with nick-translated whole insert of pILAP 4108.

partic proteinase, *Mucor miehei* aspartic proteinase, and yeast proteinase A, one in endo-thiapepsin and penicillopepsin, and none in ILAP. The lack of disulfide bridges might be one of the reasons why ILAP is so unstable above neutral pH.²⁾

As shown in Table I, codon use for ILAP has a preference for codons ending with C or G. Such codon usage has been observed in mammalian genes.²⁸⁾

Northern blot hybridization

Since pILAP 4108 neither contains the 5' coding region (preprosequence) nor the 3' noncoding region including a poly(A) tail, we tried to estimate the size of ILAP mRNA. A RNA blot hybridization of the purified poly(A⁺)RNA with a whole insert of pILAP 4108 as a probe revealed the mRNA size of ILAP to be about 1.5 kilo-nucleotides (Fig. 5). Thus the sum of the missing 5' and 3' regions of the mRNA is about 300 nucleotides.

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