Overproduction of Lactic Bacterial Enzymes and Bioactive Components

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Abstract

Recent developments in the application of molecular biology to food grade lactic acid bacteria (LAB) have shown that it could be feasible to engineer metabolic pathways to either enhance specific metabolic fluxes or to divert metabolites for the production of different or new end products. This engineering requires detailed knowledge of enzymes involved in metabolism and regulation within the targeted organism but little works have been done in this area. During biochemical and molecular characterisation of lactic bacterial enzymes, some of probiotic *Lactobacillus* and *Bifidobacterium* species were found to be very useful for food, nutraceutical and pharmaceutical industries. The enzymes are usually intracellular and the yields are very low to be useful for industrial applications. Among many enzymes and proteins of lactic bacteria studied, some of our gene cloning achievements have contributed to overproduction of lactic bacterial enzymes such as peptidases, esterases, lactases, bile salt hydrolases and linoleate isomerases for foods and nutraceuticals.

I. Introduction

Among many lactic acid bacteria (LAB), Lactobacilli and Bifidobacteria species are the most commonly used and predominant bacteria in probiotic and dairy applications. These organisms have a number of beneficial effects on host health and have been claimed to inhibit the growth of pathogens and putrefactive bacteria in the intestine by competing for nutrients and for binding sites or by producing organic acids and bacteriocins. They may also have other health-promoting effects in improving the lactose intolerance by microbial β-galactosidase (Hung et al., 2001), (2) anti-carcinogenic effect by removal of precarcinogens, or activation of the host's immune system or conjugate linoleic acid (CLA) produced by lactic linoleate isomerase (Macdonal, 2000; Wu and Lee, 2002), (3) anti-hypertensive and anti-allergenecity effects by lactic peptidases (Haileselassie et al., 1999), (4) hypocholesterolemic effect by bile salt hydrolase (Tanaka et al., 2000; Kim and Lee, 2002), and (5) synthesis of B-complex vitamins.

Due to these suggested probiotic functions, many *Lactobacillus* and *Bifidobacterium* strains from different species have been used both in pharmaceutical and dairy industry for several decades. Many lactobacilli and bifido-containing products are commercially produced worldwide, and some examples are sour cream, yogurt, cottage cheese, buttermilk, milk powder, frozen deserts, candy and pharmaceutical preparations. However, the questions on the antihypertensive, anticarcinogenic and cholesterol-lowering effects by probiotics and their underlying mechanisms are still controversial.

The peptidases are also important for cheese flavors, debitterizing protein hydrolysates, and eliminating

antigenicity problems of recombinant proteins, and esterases are also useful for the production of cheese flavors and natural fruit flavors. Commercially the combination of peptidases and esterases are also used to accelerate the ripening of various hard type cheeses.

There has been an increasing interest in the cholesterol lowering effects of fermented milk products, but not all studies reached the same conclusion and the underlying mechanism of hypocholesterolemic effect of LAB has not yet been elucidated. The hypocholesterolemic effects of some probiotics, having high BSH activities *in vitro* trials, have been demonstrated in human (Sack, 1998) as in animal (Anderson and Hanna, 1999), thus lactic acid bacteria might be responsible for the cholesterol lowering effect of probiotics but the hypocholesterolemic mechanism of probiotics based on the BSH hypothesis has not yet been sufficiently elucidated. Although commercial probiotic strains exhibit high BSH activities and some studies on BSH of *Lactobacillus* were reported (Jiang et al.,1996), biochemical and molecular aspects of BSH from Bifidobacteria of human origin at food grade level have not been studied.

CLA has also gained considerable attention because of anticarcinogenic, antioxidative, antiatherosclerotic, and immunstimulating properties (Ip et al., 1996). In addition, CLA has been reported to decrease body fat while increasing muscle and bone mass (DeLany and West, 2000; Miner et. Al., 2001). CLA is sold in health store as a muscle builder and is made by hydrogenation of oils, the process being somewhat difficult, as some of the CLA isomers are very unstable. As a result there is considerable interest in including CLA in animal feed to both improve lean production efficiency and provide value-added healthful meat and dairy products for human consumption.

The potential commercial value of CLA as a pharmaceutical and nutraceutical preparation and veterinary composition is significant and its production by microbial enzymes rather than by chemical synthesis should have advantage from the consumer point of view. However, LI enzymes from probiotic or lactic cultures have not been purified and cloned for overproduction.

To investigate the biochemical and molecular characterization of (1) peptidases: aminopeptidase (PepN) and x-prolylpeptidase (PepX), (2) esterases, (3) lactases, (4) bile salt hydrolase (BSH), and linoleate isomerase (LI) of several Lactobacillus and Bifidobacterium strains, all enzymes were purified, characterized and cloned for overproduction.

II. Materials and Methods

Bacterial strains and growth conditions.

Among many strains studied, cheese isolates, *Lactobacillus casei* LLG and *Lactobacillus rhamnosus* S93 were used for the over-production studies of peptidases and esterases. *Streptococcus thermophilus* yogurt isolate LN92 and human isolate *Bifidobacterium infantis* HL96 were used for cloning of lactases. Thirty strains of bifidobacteria were used for the BSH screening test, among which human isolate, B. *infantis* KL412 was selected for the biochemical and genetic studies. Among many *Lactobacillus* strains tested, *Lb. acidophilus* L11 was studied for linoleate isomerase (LI) in details. All growth was in MRS media at their optimum growth temperatures.

Enzyme and protein assays.

The aminopeptidase, x-prolylpeptidase and esterase were assayed spectrophotometrically with chromogenic

substrates: PepN by lysine-p-nitroaniline (Arora and Lee, 1994), PepX by gly-pro-AMC ((Habibi and Lee, 1994); caprylate-p-nitroaniline (Choi and Lee, 2001). β-Galactosidase was measured with ONPG and hydrolysis of lactose by HPLC (Hung et al., 2001). BSH activity was determined by spectrophotometrically using a chromogenic substrate, 5-amino-2-nitro-benzoic acid (ANBA)-cholate (Kim and Lee, 2002). Linoleate isomerase was also measured by isomerizaton of linoleic acid at 233 nm as well as gas chromatographically (Ogawa et al, 2001; Wu and Lee, 2002). Protein concentrations were determined with the Bio-Rad protein assay using bovine serum albumin as a standard.

Agar plate assay.

For the selection of positive blue clones containing pepN, PepX and caprylate estarase, LB agar plates containing 100ug/ml ampicillin, 50 ug/ml 5-bromo-4-chrolo-3-indolyl-substrates were used, respectively. For the screening of BSH-positive and negative strains of bifidobacteria, a selective medium for BSH activity was used. Strains were plated on MRS medium supplemented with 2 mM TDCA (1g/L). BSH activity in the medium causes hydrolysis of the soluble sodium taurodeoxycholate to deoxycholate, which is insoluble at low pH and forms a white precipitate around colonies.

Activity staining on acrylamide gel.

The crude and purified enzymes were loaded and electrophoresed in a nondenaturing 10 % (w/v) acrylamide gel, and each activity was detected by incubating the gel in buffer solutions containing the reaction mixtures at 37℃

Enzyme purification and characterization.

All enzymes were purified with ion exchange (MonoQ), gel filtration (Superose 12), affinity or hydrophobic interaction (HiTrap Phenyl FF *or* HiTrap Octyl FF) chromatographies depending on the enzymes using a FPLC system (Pharmacia). The values on molecular mass, kinetics (Km, Vmax) and inhibitors were determined.

Cloning and overexpression

The genes of pepN, pepX, and esterase derived from *Lactobacillus casei* and *Lb. rhamnosus* were cloned in *E. coli* by a shortgun cloning of a gene library of each chromosomal DNA digested with various restriction enzymes. The genes were further subcloned in pGEM72f vector or a positive selection vector, pES71 that we developed (Lee et al., 1998, US patent filed; Choi et al., 2002) and pUC19 for the overexpression. Two lactases of *Streptococcus thermophilus* and *Bifidobacterium infantis* were cloned and subcloned by our previous methods (Lee et al., 1999, US Patent filed; Hung et al., 2001). The BSH enzyme was purified, cloned and sequenced similarly, but cloning of a *Lb. acidophilus* linoleate isomerase is under investigation. All fragments generated by PCR using the gene or gene specific primers corresponding to the coding region were cloned into *Pichia pastoris* with the purpose of over-expression in yeast system using multi-copy *Pichia* expression pPIC9K vectors (Invitrogen). The over-expression levels were determined by the specific in crude lysates and activity staining. The sequence was carried out by the dideoxy chain termination method.

Antihypertenve (ACE inhibiting) peptides.

After hydrolysis of caseins and whey proteins by proteinases and peptidases produced or fermentation by those strains involved, several ACE inhibiting peptides were identified by LC/MS and characterized according to our previous methods (Haileselassie et al., 1999; Belem et al.,1999; Ahn and Lee, 2002).

Gal-oligosaccharides (GalOSs) by recombinant lactases.

Portions of 20% and 30% lactose or whey permeate were incubated with the crude extracts (25 u/ml) for different hours and hydrolysates were purified through the activated charcoal column and eluted the bound oligos by 30% ethanol and HPLC. The dried samples were identified by NMR according to our method (Hung et al., 2001). To determine the prebiotic effects, in vitro assays were carried out by intestinal bacteria, Bacteriods, 5 bifidobacteria, 4 *Lactobacillus, Clostridium, Eubacterium* and *E. coli.* grown in MRS with and without different concentration of oligos.

CLA production by *Lb. acidophilus*

The cells producing high levels of CLA were grown in MRS broth containing 5mg/ml linoleic acid at 37℃ for 24h. The washed cells (500 mg wet wt) were suspended in the enzyme reaction mixture containing 50 mg of linoleic acid 10 ml of potassium buffer, pH6.5, and BSA 0.2mg/mg linoleic acid) for 105h at 37℃. Lipids were extracted with isopropanol and hexane and saponified with 2ml of KOH. The free fatty acids were methylated with 4% HCL in methanol at 60℃ for 20 min. The organic layers were dried under nitrogen gas and dissolved in hexane for GC analysis (Ogawa et al, 2001, Wu and Lee, 2002).

III. Results and Discussion

1. Overproduction of pepN, pepX and esterase of *Lactobacillus casei/rhamnosus*

Lactobacillus casei and *Lb. rhamnosus* species which are predominant species during Cheddar cheese ripening were isolated and studied in terms of their metabolic activity, enzyme profiles and characteristics of each enzymes. The most important probiotic, *Bifidobacterium* strains also appeared to contain more active enzyme profiles than other LAB in some cases. These enzymes and cultures are very useful for not only for dairy and probiotic applications on cheese ripening, dairy flavors, lactose free products, whey sweeteners, debittering of protein hydrolysates used in food and pharmaceutical nutrition drinks, but also for the production of bioactive peptides such as ACE-inhibiting peptides, bacteriocins, CLA exhibiting anticancer and fat reducing properties as well as for lowering cholesterol levels. Aminopeptidases and x-prolylpeptidases that are very expensive ($1,000/mg) have appeared as the new emerging enzymes for pharmaceutical and analytical industries (N-terminal sequencing) to eliminate allegenecity of baby foods, recombinant proteins, and genetically modified food (GMO) crops.

The most important two enzymes, pepN and pepX consisted of less than 0.1 of total cellular proteins were genetically cloned in *E. coli* using the lamda replacement vectors, pGEM11/pGEM72f and the primers synthesis and amplication with PCR, respectively. The pepN gene was over-expressed to about 1,000 fold (50% of total cellular

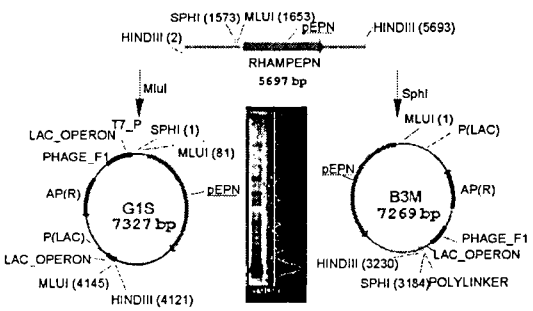


Fig. 1a. Overproduction of *L. casei* aminopeptidase (PepN , 1000 fold) in *E. coli and P. pastoris* (US patent).

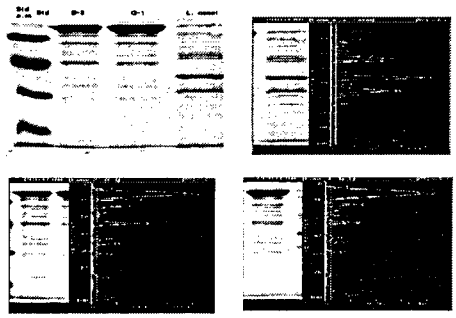


Fig. 1b. Overexpression of pepN measured by gel scanning.

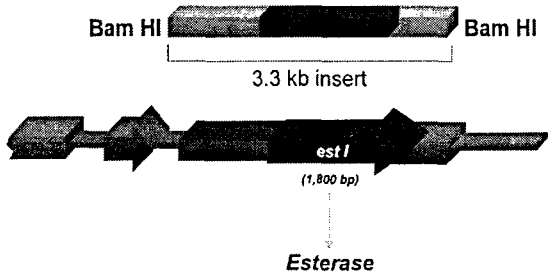
proteins) in *E. coli* (Lee and Robert, US patent file no. 60/025,172, 1998) (Fig 1a,b).The promoter sequence (pepN) of *Lactobacillus rhamnosus* was very active and efficient when expressed in *E. coli* than other exogenous transcriptional promoters such as T7 RNA polymerase. Further cloning and over-expression of the pepN was achieved into the high yielding and stable yeast, *Pichia pastoris*.

When milk and whey proteins were hydrolyzed by this enzyme in combination of proteinase (Neutrase), various bioactive peptides were produced, among which some useful bioactive peptides were identified by API-Mass spectrometry (Haileselassie et al., 1999). The peptides had a potent activity (IC50) *in vitro* but those peptides were also fed to the hypertensive, SHR rats to observe the *in vitro* effects (Unpublished).

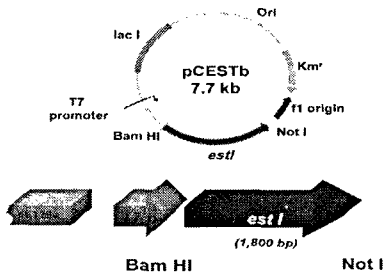
Esterases are also very important for the production of cheese flavors, natural fruit flavors such as esters and synthesizing CLA as well. A caprylate esterase of Lb. casei LLG was purified (Choi and Lee, 2001) and overexpressed (Figs a,b,c) using positive selection vector and also overproduced n yeast, P. pastoris and secreted (US patent process). This esterase has a very interesting broad specificity only for short chain fatty acids (C4 to C10) that are the most important ones as cheese flavor ingredients in cheese flavors especially for Italian types, enzyme-modified cheese, snack foods, and Cheddar cheese types. Esterases can also produce natural fruit ester compounds that are expensive. The sequence hmology of this enzyme compared with others showed the similar active site domains.

2. Overproduction of lactases of *S. thermophilus and B. infantis*

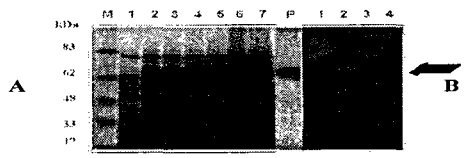
Lactases (beta-galactosidases) having strong hydrolytic (glycoside hydrolase; GH) and synthetic (galactosyltransferase; GH) activities were identified, cloned and overproduced (about 1,000 and 500 folds) in E. coli, respectively (Fig 3a,b,c). Two enzymes were also cloned into P. pastoris. The homologies of B. infantis lactases demonstrate that the beta-galI is similar to LacZ type and the beta-galIII belongs to that of LacG type in Fig 3d,e (Hung et al., 2001). The hydrolysis rates of milk lactose (4.8%) were so rapid in minutes, but no galacto-oligos were formed in this concentration that will be useful for the manufacturing of lactose-free milk products, milk fermented products, whey sweeteners and lactase enzyme itself for pharmaceutical applications. When the enzymes were incubated in high lactose solution (30%), high levels of galacto-oligosaccharides (GalOSs) were formed up to 50% (by S. thermophilus recombinant lactase) 60 % (B. infantis recombinant lactase) within 4 h that will be useful for food, nutraceutical and animal feed industries (Fig 3h). In particular, the isozyme beta-galI was very active in synthesizing GalOSs, as compared with beta-galIII, suggesting that the isozyme has a different role in GT activity. This is the first to show the different role of GH and GT activities. The structure of a major tri-oligosaccharide was



(a) Physical map of inserted esterase gene

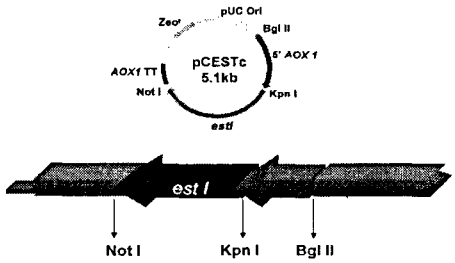


(b) Construction of E. coli expression vector

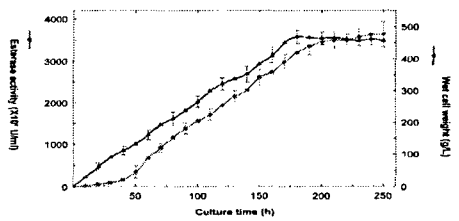


A. Lanes: M, Molecular mass standards; 1, E. coli BL21 (DE3)/pLysS bearing empty vector; 2 to 7, after 30, 60, 90, 120, 180 and 240 min induction, respectively; P, purified esterase (3 μg). B. Lanes 1 to 4, Soluble fractions after 4, 3, 2 and 1 h after induction, respectively

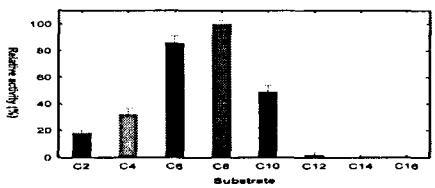
(c) Overexpression and activity staining of esteras



(d) Construction of Pichia expression vector



(e) Esterase activity and wet cell weight in P. pastoris fermentation



(f) Effect of fatty acid chain length on specificity of the recombinant esterase

L. casei CL96	*estI*	WEPGKKIHFIGHSMGGQTIRQMEEF (330)
St. epide.	*gehD*	WEPGKKIHLVGHSMGGQTIRLMEHF (165)
Dein. rad.	*lipA*	WDAAHPINLLGHSMGGQTSRMLVKL (226)
Arabidopsi.	*estD*	QLDTTKASISGHSMGGHGALTIYLR (164)
Anabaena.	*fgh*	PTEPNQTSIFGHSMGGDGALICAMK (154)
E. coli	*estA*	FPATAKKSISGHSMGGLGALVLALR (158)
Homo sapiens	*estD*	FVDFQRMSIFGHSMGGHGALICALK (161)
Sacc. cere.	*fgh*	LDFLDNVAITGHSMGGYGAICGYLK (173)
Lc. cremo.	*estA*	STKKEKNFIAGLSMGGYGAYRLALG (133)
Lc.lactis	*estA*	STKKEKNFIAGLSMGGYGAYRLALG (133)
	Consensus	NT---KISI-GHSMGG-GAL-LAL-

(g) Sequence alignment of the active site domains of with esterolytic enzymes

determined by NMR (Fig3g). GalOSs are indigestible, excellent bifidogenic factor, non-cariogenic, and are very safe products existing in human breasts milk that are desirable for the use in health foods, fortified infant formula or drinks as well as for animals as immunogenic and bifidogenic factors. Bioconversion of lactose in whey through an enzymatic process into useful products is in particular an attractive avenue for whey utilization, but the enzyme costs are a major impediment to the expansion of lactose products.

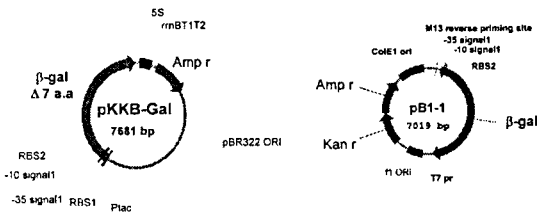


Fig. 3a. Overproduction of *Str. Thermophilus* lactase (950 fold) & *B. Infantis* lactase (500 fold) in *E. coli* and *P. pastoris* (1 US patent filed).

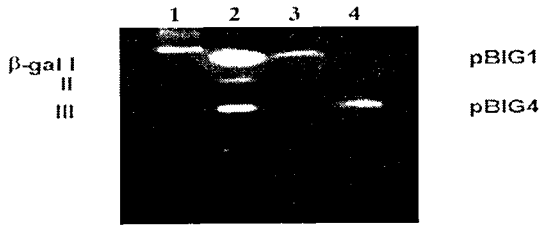


Fig. 3b. Activity staining of *B. infantis* lactases.

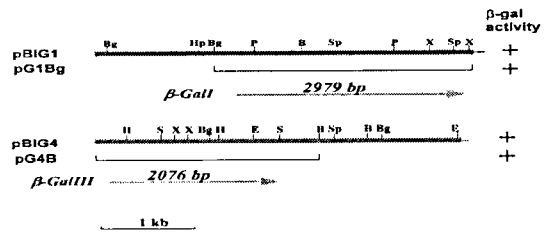


Fig. 3c. Restriction maps and ORDs *B. of infantis* lactase.

Species	No. of aa	Identical aa(%)	Similar aa(%)
B. infantis (β gall)(LacZ)	1022	100	100
S. thermophilus (lacZ)	1026	46.3	55.7
L. bulgaricus (β gal)	1007	45.8	54.8
C. acetobutylicum (bgA)	897	44.6	53
B. megaterium (bgaM)	1034	43.3	49
E. coli (lacZ)	1024	34	41.7
K. pneumoniae (lacZ)	1034	34.6	42.6

Fig. 3d. Homology between β-gall with other β-gals.

Species	No. of aa	Identical aa(%)	Similar aa(%)
B. infantis (β gall)(LacG)	691	100	100
Stearothermophilus (BgaB)	672	41.3	52
Caldicellulosiruptor sp. 14B	678	39	49.4
Thermotoga maritima	672	36.8	48.2
B. circulans (BgaA)	675	35.3	47.6
B. subtilis	687	33.1	46
Clostridium perfringens	676	36.3	46

Fig. 3e. Homology between β-gallII with other β-gals.

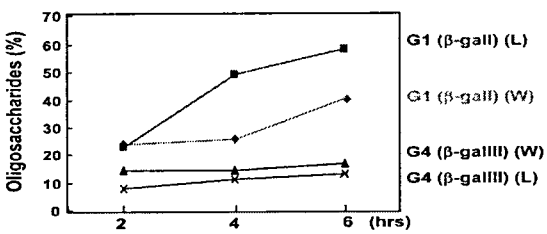
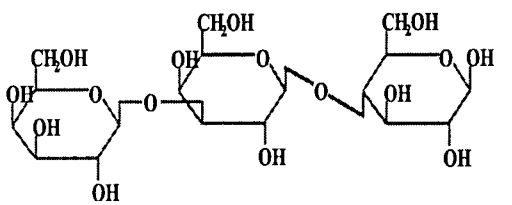


Fig. 3f. GaOS synthesis from 20%/30% lactose or whey by *B. infantis* recombinant lactase.



O-β-D-galactopyranosyl-(1-3)-*O*-β-D-galactopyranosyl-(1-4)-D-glucopyranose

Fig. 3g. Structure of a major tri-oligo by NMR.

	Expression(X)	Lactose(%)	GalOSs(%)
Crude			
B. subtilis	-	20	5
T. aquaticus	-	20	30
Bifidus (30)	-	20	32
Recombinant			
L. casei	200	20	40
S. thermophilus	950	20	45
"	-	30	50
B. infantis	500	20	55
"		30	64

* Lactose hydrolysis rate more than 90% in all recombinants

Fig. 3h. Production of galacto-oligos by different lactases.

3. Overexpression and development of BSH and LI positive probiotic strains

3.1. Bile salt hydrolase (BSH)

The study of BSH can provide a clue on cholesterol reduction by probiotic strains in their mammalian hosts. The study on the relationship between the BSH types and bile tolerance, which is one of the most important factors to be considered for the selection of probiotics, can facilitate the selection procedure of *Bifidobacterium* strains. In general, the BSH activities from Bifidobacteria were found to be higher than those of Lactobacilli (Fig 4a,b).

BSH activity staining on Native PAGE demonstrated that BSH electrophoretic mobility was different according to genus and species of the strains and two groups (A and C) of BSH enzyme were revealed (Fig. 4c). Most of bifidobacteria originated from ATCC was classified as group A, while many of commercial strains belong to group C. Each group of BSH enzyme showed two or three activity bands with its own electrophoretic mobility. This fact suggests that the possibility to form some homo- and hetero-isoenzymes combined with two different subunits α and B as reported in L. johnsonii 100 (Elkins *et al.*, 2001). The mobility of major band of group A BSH (lane 3 in Fig. 4c) was lower than that of group C (lane 2 in Fig. 4c). Group A and group C showed that BSH enzymes from the same group have some similarities in their protein structures.

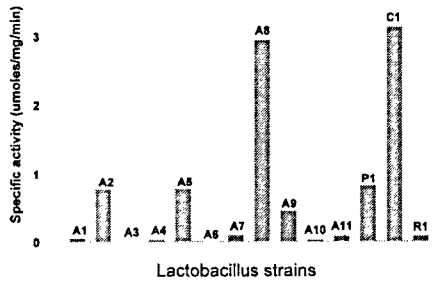
The BSH in *B. bifidum JL201* was purified 33-fold with a yield of 29 %. For group A strains the molecular masses of active fractions were between 150 and 160 kDa. For group C strains the active fractions corresponded to proteins of between 130 and 140 kDa. In many cases, the native enzymes of group A were found to be bigger than those of group C. The subunit molecular masses estimated by SDS-PAGE analyses were around 35 kDa for all BSH enzymes. This is in good agreement with those of other BSH enzymes reported previously and indicates that the native enzyme is a tetramer.

Among 4 strains tested, only *B. bifidum* ATCC 11863 had a pI of 4.42 and the other stains (*B. longum* KL507, *B. longum* KL515, and *B. infantis* KL412) had the same pI of 4.63. These values were close to the pI of 4.51 for the deduced protein of BSH from *B. longum* SBT 2928 (Tanaka *et al.*, 2000) as well as the pIs for BSHs from lactobacilli.

For the N-terminal sequencing, one strain from group A (*B. bifidum* ATCC 11863) and one strain from group C (*B. infantis* KL412) were selected. N-terminal amino acid sequencing for the protein purified from each strain resulted in the following sequences: XTGVRFSDDEGNMYFGRNLDWSFXY and XTAVRFDDGQNNMYFGRN LDWSEDY, respectively. In both cases, the Cys residue at the N-terminal of the BSH enzyme, a highly conserved area and one of the active sites of the enzyme cannot be resolved unless Cys residue is derivatized before sequencing. The N-terminal sequence of *B. bifidum* KL201 was approximately 90% identity to those of Tanaka *et al.*(2000). In addition, a conserved amino acid motif, XYFGRNLDX was highly conserved within all BSH enzymes reported. All purified BSHs were strongly inhibited by SH enzyme inhibitors, suggesting that these enzymes are thiol enzymes. The results of expression and sequencing of BSH are submitted for the patent protection. After cloning of the BSH genes from *B. infantis* JL201 in *E. coli* and hybridization with the probe synthesized, the BSH clones were analyzed (Fig 4d,e) and completely sequenced.

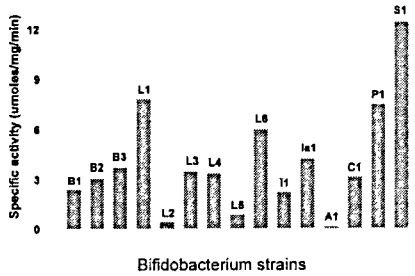
3.2. Linoleate isomerase (LI)

To investigate the possibility of LI enzyme that can produce CLA for the industry application, detailed works were carried out to study: (1) the growth and enzyme production, (2) purification, (3) characterization and function of this enzyme by GC analyses. Among six strains of *Lactobacillus acidophilus, L. fermentum and L. reuteri tested,*



❖ 1 Unit : the amount of enzyme which liberated 1 μmol of ANBA from the substrate per minute.

Fig. 4a. Screening of BSH active strains from lactobacilli.



❖ 1 Unit : the amount of enzyme which liberated 1 μmol of ANBA from the substrate per minute.

Fig. 4b. Screening of BSH active strains from *Bifidobacteria.*

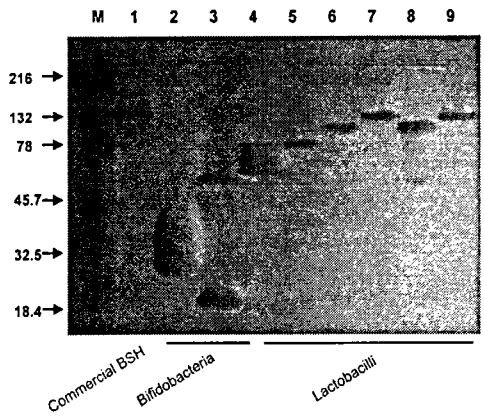


Fig. 4c. Activity staining on a native PAGE.

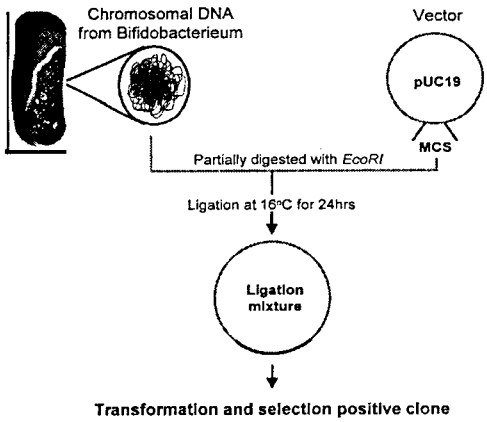


Fig. 4d. Cloning of *B. infantis bsh* clones.

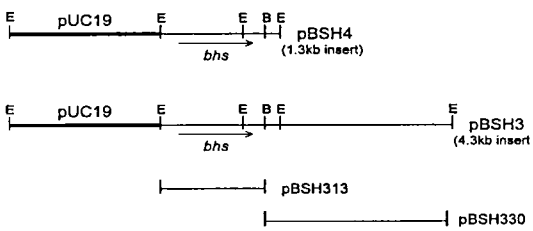


Fig. 4e. Selection of *B. infantis bsh* clones.

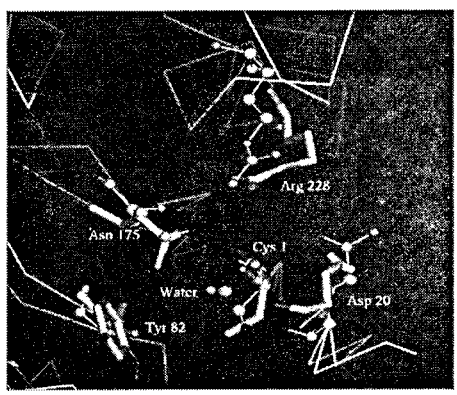


Fig. 4f. Proposed active sites of PVA.

Lactobacillus acidophilus L11 that produced the good enzymatic activity was chosen to characterize the LI enzyme from the cell grown in MRS broth containing 5 mg/ml linoleic acid (Fig.5). *Lactobacillus reuteri* appeared to be better than others in terms of CLA production.The molecular mass of this enzyme was estimated to be 72 kDa by SDS-PAGE and this enzyme has two identical subunits with total mass of 144 kDa on native-PAGE as well as gel

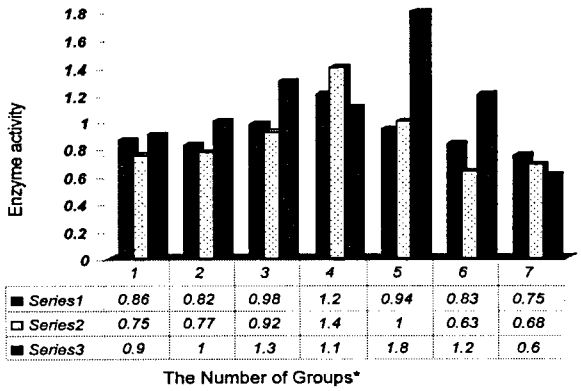


Fig. 5. Screening of LI active strains from lactobacilli.

Table 5. Purification of LI from *L. acidophilus*

Steps	Total protein (mg)	Total activity (units)	Specific activity (u/mg)	Purification fold (x)	Yield (%)
1. Crude extract	131.94	113.47	0.86	1.00	100
2. Ammonium sulfate ppt.	112.15	109.64	0.98	1.14	96.60
3. Ion-exchange I	1.6	75.00	46.88	42.60	66.10
4. Ion-exchange II	0.8	72.10	90.13	104.80	63.54
5. Gel filtration	0.2	17.82	104.83	121.87	15.70

*One unit of enzyme activity is defined as the amount of enzyme required to catalyze 1 n mole of linoleic acid/min.

filtration. The pH and temperature optima were 7.0 and 37°C, respectively. The Km and Vmax for linoleic acid were 14.9 μM and 17.1 μM/mg/min, respectively. The LI protein of *Lactobacillus LI1 was* microsequenced and the N-terminal peptide sequence (20 amino acid) was synthesized to prepare and to screen the genomic library. The molecular characterization was being carried out to confirm the role of LI and to serve as an enzyme for producing CLA.

IV. Conclusions

1. For the first time, minute quantity but very active lactic bacterial enzymes were overproduced in *E. coli and P. pastoris* genetically for the production of various bioactive components.
2. Aminopeptidase (pepN) and x-prolylpeptidase (pepX) of *L. casei* species that are useful for food, nutraceutical and pharmaceutical applications were overproducedup to 1,000 folds (about 50 % of total cellular proteins).
3. A caprylate esterase that has broad specificity for short chain fatty acids (C_4-C_{10}) was overproduced (1,000 folds) in *E. coli or P. pastoris* and secreted for various flavors and inter-esterification applications.
4. Two lactases of *S. thermophilus and B. infantis* were overproduced and showed very active hydrolytic (GH) and galactosyltransferase (GT) activities. The isozymes seemed to have a different role in GT activity.

5. Biochemcal, phylogenetic and molecular characterization of bile salt hydrolase (BSH) of *B. infantis* were studied in details for the possible role in cholesterol lowering effects. Linoleate isomerase (LI) from L. acidophilus was purified, characterized and the cloning is under investigation.

V. References

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