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Growth promotion of *Bifidobacterium animalis* by bovine milk proteose-peptone

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Summary — The industrial strain *Bifidobacterium animalis* was used as assay organism to evaluate bifidobacterial growth-promoting activity of bovine milk proteose-peptone. This proved to be a better growth-promoting factor than bovine casein. The bifidogenic activity was found mainly in the proteose-peptone hydrophobic fraction containing component 3, although the glycan moiety was a weak growth-promoter. Proteose-peptone digests by various proteolytic enzymes caused great enhancement of *B animalis* growth, particularly the Pronase digest. Size-exclusion chromatography of digests showed that the more active peptides had a molecular mass distribution of 1000 to 5000 Da.

proteose-peptone / component 3 / *Bifidobacterium animalis* / growth stimulation / proteolysis / milk

Résumé — Activité stimulante des protéose-peptones de lait bovin sur la croissance de *Bifidobacterium animalis*. *La souche industrielle* Bifidobacterium animalis *a été utilisée pour évaluer l'activité stimulante des protéose-peptones du lait bovin. Elles se sont montrées de meilleurs facteurs bifidogéniques que la caséine. L'activité bifidogénique a été principalement trouvée dans la fraction hydrophobe des protéose-peptones contenant le composant-3, bien que la partie glycannique du composant-3 n'ait qu'une faible activité stimulante. L'hydrolyse des protéose-peptones par des enzymes protéolytiques provoque une forte augmentation de la croissance de* B animalis, *tout particulièrement avec l'hydrolyse pronasique. L'étude des hydrolysats par chromatographie d'exclusion stérique montre que les peptides les plus actifs ont une taille comprise entre 1000 et 5000 Da.*

protéose-peptone / composant-3* / Bifidobacterium animalis / *stimulation de la croissance / protéolyse / lait

INTRODUCTION

Bovine milk proteose-peptone represents about 10% of the whey protein and is a heat-stable and acid-soluble fraction which can be separated into two groups (Pâquet, 1989). The first includes polypeptides resulting from proteolysis of casein by endogenous proteinase in milk among which β-CN-5P (f1-105/107; 12 441 Da), β-CN-4P (f1-28; 3478 Da), and β-CN-1P (f29-105/107; 8981 Da) are N-terminal fragments of β-casein (Eigel *et al,* 1984). The second group can be separated from the first by hydrophobic interaction liquid chromatography (Pâquet *et al,* 1988; Girardet *et al,* 1991) and contains three principal glycoproteins with apparent M_r of 11 000, 19 000, and 29 000 Da. The primary structure of component 3 corresponding to the 29 000-Da glycoprotein has been recently determined (Sørensen and Petersen, 1993) and shown to contain 135 amino acid residues. Two O-linked (Tyr16 and Tyr86) and one N-linked (Asn77) glycans are bound on the polypeptide chain. This carbohydrate moiety represents 17–18% of the component 3 fraction (Kanno, 1989; Girardet *et al,* 1993). Moreover, the 19 000-Da glycoprotein was identified as the 54–135 C-terminal fragment of component 3 (Sørensen and Petersen, 1993).

Component 3 has interesting functional properties, particularly its important emulsifying capability (Shimizu *et al,* 1989) and its biochemical role in the inhibition of milk's spontaneous lipolysis (Cartier *et al,* 1990; Girardet *et al,* 1993). Recently, the mitogenic activity of a hydrophobic fraction containing component 3 on DNA synthesis in MARK 3 hybridoma was shown (Mati *et al,* 1993).

The growth-promotion of *Bifidobacterium bifidum* var *pennsylvanicus* has been extensively studied (György and Rose, 1955; Kehagias *et al,* 1977; Bezkorovainy and Topouzian, 1981). Human milk proteose-peptone contains growth-promoting factors for this strain (Bezkorovainy *et al,* 1976), but there is very little such growth-promoting activity in the bovine milk proteose-peptone. However, this strain may not accurately reflect the biochemical response of the genus *Bifidobacterium* to growth factors. Moreover, this model strain is uncommon in the intestinal tract and is considered as a mutant, since it requires exogenous N-acetyl-glucosamine for cell wall synthesis (Poch and Bezkorovainy, 1988). Consequently, much of the growth-promoting activity has been attributed to the N-acetyl-glucosamine-containing oligosaccharides and glycoproteins. Human milk proteose-peptone contains more carbohydrates (45% according to Bezkorovainy *et al,* 1976) than bovine milk proteose-peptone. No study has been published on bovine milk proteose-peptone promotion of bifidobacterial growth, although growth-promoting activity of *Bifidobacterium* species by bovine or human whey proteins and caseins was tested (Petschow and Talbott, 1990, 1991).

It is now well-established that bifidobacteria strains cannot grow in synthetic media, but require unknown factors for optimal growth (Poch and Bezkorovainy, 1988). The purpose of this work was to investigate the growth-promoting activity of bovine milk proteose-peptone for *B animalis,* one of the commonly used strains in dairy industry (Roy and Ward, 1993).

MATERIALS AND METHODS

Materials and reagents

The fast protein liquid chromatography (FPLC) system and TSK-Phenyl-5PW (21.5 x 150 mm) column were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The HPLC L6200 apparatus was from Merck (Darmstadt, Germany) and the TSK-2000 SW column from Pharmacia. Bio-gel P2 was from Bio-Rad Laboratories (Richmond, VA, USA). An ultrafiltration cell 52-model

and PM-10 membrane (cut-off 10 000 Da) were purchased from Amicon (Danvers, MA, USA). Dialysis tubing was cellulose ester (cut-off 500 Da; Medicell International, London, UK). Enzymes used were: immobilized neuraminidase (EC.3.2.1.18) of *Clostridium perfringens*, type VI-A (Sigma Chemicals Co, Saint-Louis, MO, USA), Pronase E (EC.3.4.24.4) of *Streptomyces griseus* (Merck, Darmstadt, Germany), N-tosyl-L-phenylalanine-chloromethylketone (TPCK)-treated trypsin (EC.3.4.21.4) and α-chymotrypsin (EC.3.4.21.1) both from bovine pancreas and attached to beaded agarose (Sigma). Amino acids (Lys, His, Arg, Asp, Thr, Ser, Glu, Pro, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Phe, Trp), N-acetylglucosamine, lactose, lactulose were purchased from Sigma, and the brain heart infusion (BHI) from Difco Laboratories (Detroit, MI, USA).

Fractionation of milk proteins

Whole casein was prepared by acid precipitation of Holstein cows' raw skim milk at pH 4.6 by 1 mol/l-HCl and soluble whey proteins were dialyzed and freeze-dried. Proteose-peptone extracts were prepared after bulk skim milk treatment by heating (95°C, 30 min) and acidification with 1 mol/l-HCl at pH 4.6 (Pâquet *et al*, 1988).

Fractionation of proteose-peptone

Proteose-peptone fractionation was performed using a TSK-Phenyl-5PW column connected to the FPLC system (Girardet *et al*, 1991). Sample (150–200 mg) was loaded on the column equilibrated in 0.8 mol/l-NaH_2PO_4 buffer (pH 6.8) at 25°C. Flow rate was 6 ml/min and detection was measured at 280 nm. An isocratic elution of 20 min in 0.8 mol/l-NaH_2PO_4 was performed to collect the non-hydrophobic fraction of proteose-peptone (NHF). After a second isocratic step of 18 min at 0.15 mol/l-NaH_2PO_4, the low hydrophobic fraction of proteose-peptone (LHF) was eluted. Last, the highly hydrophobic fraction of proteose-peptone (HHF) containing component 3 was obtained in pure water (isocratic elution of 20 min in 0 mol/l-NaH_2PO_4). After dialysis, each fraction was freeze-dried. Between each chromatography step, the column was washed by injection of 2.5 ml of 200 mmol/l-NaOH.

Proteose-peptone was also separated by ultrafiltration using a membrane with a nominal molecular mass cut-off of 10 000 Da. Filtrate and retentate fractions were referred as low and high M_r fractions respectively.

Neuraminidase treatment of high hydrophobic fraction of proteose-peptone

Desialylation was carried out according to the method of Coddeville *et al* (1989). HHF (10 mg) in 0.1 mol/l-citrate buffer (pH 5.5), was treated at 37°C for 24 h with 5 units (one unit will liberate 1 μmol of N-acetylneuraminic acid per min at pH 5 at 37°C, using NAN-lactose as substrate) of immobilized neuraminidase.

Preparation of glycopeptides

Proteolysis of proteose-peptone and purification of glycopeptides were performed according to Montreuil *et al* (1987). The proteose-peptone (1 g) in 10 mmol/l-calcium acetate buffer (pH 8) was treated with Pronase E (enzyme:substrate, 1:50) (4 000 000 PU/g, one unit (PU) will hydrolyse casein to produce color equivalent to 1 μmol of tyrosine per 10 min at pH 7.4 at 40°C, color by Ciocalteu reagent), at 37°C for 6 h. Enzymic activity was stopped by adding acetic acid to pH 4.5. Cold ethanol (9 vol, −10°C) was then added to precipitate glycopeptides. This fraction was then dissolved in calcium acetate buffer and hydrolysis with Pronase was performed twice more. Then, trichloroacetic acid to a final concentration of 12% (w/v) was added to precipitate excess enzyme and, after adjustment to neutral pH by NaOH, glycopeptides were desalted by passing through a Bio-gel P2 (1.5 x 53 cm) column. Those fractions, which stained at 80°C for 5 min with 0.2% (v/v) 3,5-dihydroxytoluol in 20% (v/v) H_2SO_4, were pooled and freeze-dried.

Proteolytic treatments of proteose-peptone

Freeze-dried proteose-peptone (50 mg) in 25 ml of a mixture of 43 mmol/l-HCOOH and

57 mmol/l-NH_3 was treated with 10 N-α-benzoyl-L-arginine-ethylester (BAEE) units of trypsin or 10 N-acetyl-L-tyrosine-ethylester (ATEE) units of α-chymotrypsin both attached to beaded agarose. Hydrolysis occurred during 2.5 h at 37°C under gentle mixing. The reaction was stopped by centrifugation (1800 *g*, 5 min). For double hydrolysis the second step is then realized under the same conditions. Afterwards, the supernatant was evaporated under partial vacuum and freeze-dried in order to remove ammonium formate.

Digestion by Pronase E (4 000 000 PU/g) was performed during 2.5 h at 37°C in the same ammonium formate buffer (pH 8.5) (enzyme:substrate, 1:50). The reaction was stopped by adding acetic acid to pH 4.5. The product was ultrafiltered (cut-off 10 000 Da) to remove Pronase and the permeate was evaporated under partial vacuum and freeze-dried.

Analytical methods

Protein concentration was determined by the procedure of Lowry *et al* (1951), with bovine serum albumin as standard.

The degree of hydrolysis of each fraction was measured in triplicate from the amount of free amino groups with the trinitrobenzene sulphonic acid spectrometric method (Church *et al*, 1985), using glycine as the standard.

The molecular mass distribution profiles of enzymic hydrolysates were determined by high performance size-exclusion chromatography (HPSEC; Vijayalakshmi *et al*, 1986). Protein standards (Serva Fine Biochemicals, Heidelberg, Germany) were α-lactalbumin (14 200 Da), trypsin inhibitor (6500 Da), melittin (2847 Da), bradykinin (1060 Da), and Pro-Leu-Gly-Pro-D-Arg (795 Da).

Bacterial strain and growth conditions

The *Bifidobacterium* strain, kindly supplied by Chr Hansen (Copenhagen, Denmark), has been industrially employed to prepare fermented milks. The fructose-6-phosphate-phosphoketolase (specific enzyme of bifidobacteria) test was done as described by Scardovi (1986) and the strain was genetically identified by its ribosomal RNA gene restriction patterns (Grimont and Grimont, 1986) as *Bifidobacterium animalis* ATCC 27536 (result not shown). The microorganism was routinely propagated and subcultured three times before experiments in BHI broth (Romond *et al*, 1980). The standard bacterial inoculum was prepared from cultures of 24 h at 37°C. Bifidobacteria were centrifuged 15 min at 5000 *g*, suspended in 0.9% (w/v) NaCl and 0.1% (w/v) peptone (Collins and Hall, 1984), then standardized to a turbidity of 0.65 measured at 550 nm (approximately 10^9 cells/ml; Roy *et al*, 1990).

Bifidobacterial growth promotion assay

Growth of microorganisms in the presence of potential growth promoters was based in part on the method of Poch and Bezkorovainy (1991), using synthetic Garches medium (Romond *et al*, 1980).

After compounding with or without the growth promoters to be tested (1 mg/ml), the test medium was sterilized by autoclaving (110°C, 20 min). The medium (10 ml) was inoculated with 0.1 ml of the standard bifidobacterial inoculum and incubated aerobically at 37°C for 24 h without agitation. Growth was determined by measurement of turbidity at 635 nm (Proulx *et al*, 1992) (turbidity of 0.15 ± 0.05 for synthetic Garches medium alone). In order to standardize the results, all growth experiments in the synthetic Garches medium were accompanied by growing the microorganism in BHI broth (reference standard; turbidity of 1.4 ± 0.15), because growth varied from day to day (Poch and Bezkorovainy, 1988). The formula: growth promotion (%) = [A test medium – A synthetic medium / A reference medium – A synthetic medium] x 100, was used to express growth, where A synthetic medium corresponded to the turbidity obtained with the Garches medium alone. All assays were done three times in duplicate.

Statistical methods

Multiple comparisons were made using a multiple range test based on confidence intervals and differences between sample means.

RESULTS

Growth promotion activity of milk protein fractions

Casein, whey proteins and proteose-peptone, prepared from bovine milk were tested for *B animalis* growth promotion activity (table I). All significantly enhanced growth in comparison with the synthetic medium alone (0.15 ± 0.05%). Both whey proteins and proteose-peptone were better growth-promoting factors of *B animalis* than casein ($P < 0.01$).

Distribution of activity among proteose-peptone fractions

Proteose-peptone was divided in terms of its hydrophobic behavior (fig 1). NHF, LHF and HHF were identical to fractions I, II+III+IV and V respectively, isolated in our earlier work (Girardet *et al*, 1991). So, NHF was composed by hydrophilic peptides or polypeptides. LHF contained principally β-CN-5P (f1-105/107), and the high hydrophobic fraction (HHF) contained mainly component 3. The *B animalis* growth promoting activities of NHF and LHF were not significantly different ($P > 0.05$), but lower ($P < 0.01$) than those of proteose-peptone and HHF (table II).

Moreover the low and high M_r fractions of proteose-peptone displayed significant activity, especially the latter ($P < 0.01$).

Therefore, the activation capability of the proteose-peptone could be mainly due to fractions containing hydrophobic compo-

Table I. *Bifidobacterium animalis* growth-promoting activity of bovine milk protein fractions.
Activité stimulante des fractions protéiques du lait bovin sur la croissance de Bifidobacterium animalis.

Milk protein fraction (1 mg / ml)	*Growth-promoting activity* (%)*
Casein	6.0 ± 1.2 a†
Whey proteins	9.5 ± 1.8 b
Proteose-peptone (PP)	10.9 ± 2.1 b

* Defined in text. † Different letters show significant difference ($P < 0.01$).
**Défini dans le texte. † Des lettres différentes montrent une différence significative ($P < 0,01$).*

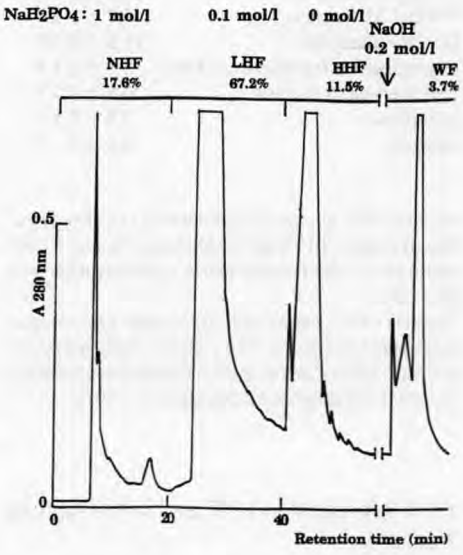

Fig 1. Fractionation of proteose-peptone by semi-preparative hydrophobic interation FPLC in a TSK-Phenyl 5PW column (method adapted from Girardet *et al*, 1991). Nitrogen content of each collected fraction was determined by the Kjeldahl method. NHF, non-hydrophobic fraction; LHF, low hydrophobic fraction; HHF, high hydrophobic fraction; WF, wash fraction.
Fractionnement des protéose-peptones par FPLC d'interactions hydrophobes semi-préparative sur colonne TSK-Phenyl 5PW (méthode adaptée d'après Girardet et al, *1991). Le contenu en azote de chaque fraction collectée est déterminé par la méthode de Kjeldahl. NHF : fraction non hydrophobe ; LHF : fraction faiblement hydrophobe ; HHF : fraction fortement hydrophobe ; WF : fraction de lavage.*

Table II. *Bifidobacterium animalis* growth-promoting activity of proteose-peptone fractions. *Activité stimulante des fractions de protéose-peptones sur la croissance de* Bifidobacterium animalis.

Proteose-peptone fraction (1 mg / ml)	*Growth-promoting activity * (%)*
Proteose-peptone (PP)	10.9 ± 2.1 a†
NHF	7.3 ± 1.8 b
LHF	7.4 ± 1.6 b
HHF	11.8 ± 2.3 a
PP high M_r fraction	16.2 ± 2.3 c
PP low M_r fraction	3.7 ± 0.5 d
Desialylated HHF	11.9 ± 2.5 a
Glycopeptide fraction from HHF	6.6 ± 2.1 b
N-Acetyl-glucosamine	0.8 ± 0.2 e
Lactulose	0.2 ± 0.1 f
Lactose	0.2 ± 0.1 f

Legend: NHF, non-hydrophobic fraction; LHF, low hydrophobic fraction; HHF: high hydrophobic fraction. * Defined in text. † Different letters show significant difference ($P < 0.01$).
*Légende : NHF : fraction non hydrophobe ; LHF : fraction faiblement hydrophobe; HHF : fraction fortement hydrophobe. * Défini dans le texte. † Des lettres différentes montrent une différence significative (P < 0,01).*

nent 3 (present in HHF and in the high M_r fraction).

Growth response to carbohydrate promoters

To determine the role of component 3 sialic acids on the activation of the *B animalis* growth, neuraminidase treatment of HHF was performed, but no change was observed after desialylation ($P > 0.05$). The activity of the glycopeptide fraction was significantly lower than the HHF and proteose-peptone activities ($P < 0.01$; table II). N-Acetyl-glucosamine and lactulose, known as growth promoters for bifidobacteria, showed no growth promotion of *B animalis* (table II).

Enzymic digests of proteose-peptone

The M_r distribution of the proteose-peptone digests were related approximately to the degree of hydrolysis (table III) and also to the growth-promoting activity of *B animalis* (table IV). PP^T and PP^C consisted mainly of large peptides (5000–10 000 Da) whereas PP^{TC} and PP^{CT}, obtained by double trypsin-α-chymotrypsin (PP^{TC}) or α-chymotrypsin-trypsin (PP^{CT}) hydrolysis, contained equal amounts of large (5000–10 000 Da) and small (2000–5000 Da) peptides. PP^{TC} and PP^{CT}, with more amino groups than PP^T and PP^C, were better growth-promoting factors than the simple hydrolysates (table IV). Pronase digestion of proteose-peptone (PP^P) gave the highest degree of hydrolysis, and contained only small peptides (< 5000 Da). PP^P was a good stimulant of *B animalis* growth (table IV).

By dialysis of PP^T (cut-off 500 Da), a significant increase of the activity was observed ($P > 0.05$; table IV).

In order to test the effect of free amino acids on the growth promotion of *B animalis*, a mixture of 18 amino acids was added to the Garches medium. Amino acids were added at 50 mg/l except for cysteine (6.25 mg/l). The growth-promoting activity was weak.

DISCUSSION

Proteose-peptone represents a milk fraction that still remains to be valorized in dairy industry. In this study, we have tested its growth-promoting activity on *B animalis*, a strain commonly used in fermented milk production, contrary to label information (Biavati *et al*, 1992; Roy and Ward, 1993).

Table III. Hydrolysis of proteose-peptone by various proteases and molecular mass distribution of peptides produced.
Hydrolyses protéasiques des protéose-peptones et distribution de la masse moléculaire des peptides produits.

Proteose-peptone digest	*Hydrolysis degree mmol / l equivalent Gly*	*M_r distribution of peptides* (%)*				
		>10 000 Da	*5000-10 000 Da*	*2000-5000 Da*	*1000-2000 Da*	*< 1000 Da*
PP	0.40	95.0	5.0	–	–	–
PP^T	1.30	12.0	53.0	35.0	–	–
Dialyzed PP^T	0.95	13.0	53.0	34.0	–	–
PP^C	1.20	21.5	59.0	19.5	–	–
PP^{TC}	1.99	5.5	48.0	45.0	–	1.5
PP^{CT}	2.20	6.4	45.8	43.8	–	4.0
PP^P	4.30	–	–	67.5	24.5	8.0

PP, proteose-peptone; PP^T or PP^C, trypsin or α-chymotrypsin hydrolysate; PP^{TC} or PP^{CT}, trypsin-α-chymotrypsin or α-chymotrypsin-trypsin double hydrolysate; PP^P, pronase hydrolysate. * Molecular mass distribution is calculated from the integration of the surface of the chromatogram. Results were expressed in percentage of the total surface.
*PP : proteose-peptones ; PP^T ou PP^C : hydrolysat trypsique ou α-chymotrypsique ; PP^{TC} ou PP^{CT} : double hydrolysat trypsique-α-chymotrypsique ou α-chymotrypsique-trypsique ; PP^P : hydrolysat pronasique. * La distribution de la masse moléculaire est estimée à partir de l'intégration de la surface totale du chromatogramme. Les résultats sont exprimés en pourcentage de la surface totale.*

Bovine whey proteins and proteose-peptone proved to be better growth-promoting factors of *B animalis* than casein (table I), and these results are in good agreement with the work of Petschow and Talbott (1990). They showed that the growth-promoting activity of several strains (*B bifidum, B infantis, B breve, B longum*) is contained in the acid whey rather than in the acid casein fraction, concluding that cow milk contains heat-stable growth promoting factors in whey. We believe that these factors could be present in the heat-stable and acid-soluble fraction called proteose-peptone.

Although the presence of the growth-promoting activity in both the non-protein (< 10 000 Da; low M_r) and the protein (high M_r) fractions of whey has been reported (Petschow and Talbott, 1991), we did not find much activity in the low M_r fraction of proteose-peptone. This fraction could contain small hydrophilic peptides such as β-CN-4P (f1-28). The two principal compounds β-CN-5P (f1-105/107) and component 3 (in an aggregate state in milk > 100 000 Da; Pâquet, 1989) remaining in the protein proteose-peptone fraction after ultrafiltration showed a good growth-promoting activity.

The glycan moiety of component 3 contains fucose, galactose, mannose, N-acetylgalactosamine, N-acetylglucosamine and N-acetyl-neuraminic acid (Kanno, 1989; Girardet *et al,* 1994). Moreover, the component 3 N-linked glycan (bounded to Asn77; Sørensen and Petersen, 1993) seems to be a biantennary N-acetyllactosamine-type structure (Girardet *et al,* 1994). So, the glycan moiety has an heterogeneous structure composed of N- and O-linked chains. N-acetyl-neuraminic acids bound at the free extremity of the glycan

Table IV. *Bifidobacterium animalis* growth-promoting activity of proteose-peptone digests *Activité stimulante d'hydrolysats de protéose-peptones sur la croissance de* Bifidobacterium animalis.

Proteose-peptone fraction (1 mg/ml)	*Growth-promoting activity * (%)*
PP	10.9 ± 2.1 a†
PP^T	26.3 ± 2.7 b
Dialyzed PP^T	33.7 ± 3.6 c,h
HHF^T	28.7 ± 3.1 b,h
PP^C	21.3 ± 2.4 d
PP^{TC}	46.8 ± 4.2 e
PP^{CT}	38.3 ± 3.9 c
PP^P	72.3 ± 5.8 f
Free amino acids ‡	7.2 ± 1.1 g

PP, proteose-peptone; HHF, high hydrophobic fraction; PP^T or PP^C, trypsin or α-chymotrypsin hydrolysate; PP^{TC} or PP^{CT}, trypsin-α-chymotrypsin or α-chymotrypsin-trypsin double hydrolysate; PP^P, Pronase hydrolysate. * Defined in text. † Different letters show significant difference ($P < 0.01$). ‡ Free amino acids are a mixture of Lys, His, Arg, Asp, Thr, Ser, Glu, Pro, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Phe, Trp.

*PP : protéose-peptones ; HHF : fraction fortement hydrophobe ; PP^T ou PP^C : hydrolysats trypsique ou α-chymotrypsique ; PP^{TC} ou PP^{CT} : double hydrolysat trypsique-α-chymotrypsique ou α-chymotrypsique trypsique ; PP^P : hydrolysat pronasique. * Défini dans le texte. † Des lettres différentes montrent une différence significative (P < 0,01). ‡ Acides aminés libres constitués d'un mélange de Lys, His, Arg, Asp, Thr, Ser, Glu, Pro, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Phe, Trp.*

chains may protect the glycan moiety against hydrolysis by the recurrent glycolytic system of the bifidobacteria strains (Nicolai and Zilliken, 1972). Indeed, most of the species of *Bifidobacterium* do not have neuraminidase (Desjardins *et al*, 1990). However, in spite of the removal of the glycan residues after enzymic desialylation of HHF, the growth of *B animalis* was not increased. In the case of *B bifidum* var *pennsylvanicus*, the oligosaccharides tested are good growth-promoting factors only after neuraminidase treatment (Nicolai and Zilliken, 1972; György *et al*, 1974). N-Acetyl-glucosamine contained in these oligosaccharides is available after hydrolysis of the terminal sialic acids. However, it is known that N-acetyl-glucosamine is only needed for the growth of *B bifidum* var *pennsylvanicus*; the other species do not take up this glycan residue. The growth-promoting factors contained in the proteose-peptone should be distinguished from the oligosaccharide and glycoprotein activations of the mutant *B bifidum* var *pennsylvanicus*.

Glycopeptides purified after Pronase treatment of proteose-peptone are generated by proteolysis of component 3, the only known glycosylated compound of proteose-peptone (Pâquet, 1989). Because of the weak growth-promoting activity of these glycopeptides, the growth factors were most likely peptides.

The enzymic specificity of proteinases affects the hydrolysis kinetics of proteose-peptone and the M_r distribution of peptides produced. The trypsin digestion was greater than the α-chymotrypsin one. Basic amino acids (Lys and Arg) located mainly at the surface of the molecule are more accessible than aromatic amino acids and are more plentiful in the primary structure of component 3 (Sørensen and Petersen, 1993).

After proteose-peptone digestion by endopeptidases, the growth of *B animalis* was more active. It is reported that bifidogenic activity is increased by enzymic hydrolysis especially of bovine casein (Kehagias *et al*, 1977; Poch and Bezkorovainy, 1988; Proulx *et al*, 1992). Moreover, the growth of *B animalis* was enhanced when the degree of hydrolysis was increased (by trypsin-α-chymotrypsin double digestion). Pronase, a non-specific proteolytic system composed of various types of endopeptidases and exopeptidases, can generally proceed to the level of single amino acids. The large specificity of Pronase explains the high degree of proteose-peptone hydrolysis and

the large amount of small peptides generated (all peptides < 5000 Da). In general, the growth-promoting activity was correlated with the ratio of medium (2000–5000 Da) and small (1000–2000 Da) peptides. Proulx *et al* (1992) have shown that bovine casein peptides < 2000 Da are the best activators of the growth of *B infantis, B breve, B longum*, and *B adolescentis*. Peptides isolated from cellular filtrate of *Lactobacillus casei* (948–2319 Da) enhanced the growth of *B infantis* and *B breve* (Cheng and Nagasawa, 1984).

The deficiency of free amino acids in the growth-promoting activity could be explained by possible competition in free amino acid transport systems of *B animalis*.

Dialysis of PP^T resulted in important decrease of degree of hydrolysis while the modification of M_r distribution of peptides varied by only 1% (table III). This difference in degree of hydrolysis of dialysed PP^T could be explained by the elimination of ammonium (ammonium formate buffer) during dialysis. Moreover, elimination of compounds < 500 Da by dialysis of the proteose-peptone trypsin hydrolysate (PP^T) resulted in an enhancement of the *B animalis* growth. Poch and Bezkorovainy (1991) showed that dialysis of κ-casein digest (cut-off 1000 Da) increased the activity of the dialyzed fraction. Moreover, no correlation was observed between the growth-promoting activity and the proportion of very small peptides (< 1000 Da), as suggested by the results obtained with PP^{TC} and PP^{CT}. Therefore, peptides produced by enzymic hydrolysis of proteose-peptone (or of bovine casein; Poch and Bezkorovainy, 1988) seem not to be simply amino acid suppliers for the growth of *B animalis*. Peptides with a M_r range of 1000 to 5000 Da seem to be a better nitrogen source of growth factors than very small peptides (< 1000 Da) or free amino acids. This result agrees with those reported previously (Poch and Bezkorovainy, 1988; Proulx *et al*, 1992) and with those of Ibrahim *et al* (1993) who found no growth promotion of *B longum* with κ-casein trypsin hydrolysates (< 500 or > 6000 Da). Growth activators were located in peptides having M_r between 1000 and 6000 Da. Meanwhile, Cheng and Nagasawa (1984) showed that the upper size limit for peptide transport through lactic streptococcal membranes is five or six residues. Accordingly, extracellular and intracellular peptidases of bifidobacteria could play an important role in the peptide uptake for their growth (Desjardins *et al*, 1990).

To conclude, bovine milk proteose-peptone appears to contain factors able to promote the growth of this industrial strain of bifidobacteria. These factors seem to be associated with fractions containing component 3. The carbohydrate moiety of component 3 has a weak efficiency on the growth activation that seems to be due to the peptide. Moreover, proteolytic digestion of proteose-peptone or HHF considerably increases this stimulation. The most efficient peptides seemed to be of hydrophobic nature and may have a M_r distribution of 1000 to 5000 Da; both size and origin of peptides could be important for its growth promotion activity. However, the eventual presence of unknown substances bound to the hydrophobic peptides must be considered. Separation and characterization of growth-promoting peptides are now in progress.

In dairy industry, the increase of the ratio of proteose-peptone (previously treated by proteolytic enzymes) in cow milk-based formulae could result in an enhancement of the *B animalis* growth.

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