water samples A and B using gas chromatography (Wilson Testing Laboratories, Shamokin, PA, USA) yielded values of 0.07 and <0.005 mg/100 ml of P and DMP, respectively [22]. Peak identities were confirmed by comparing the retention times to those of the standards. Additional confirmation of peak identity was achieved by spiking the samples with small amounts of the known compounds resulting in the enlargement of existing peaks. A number of additional AEOC-reactive impurities were also observed.

4. Conclusion

It has been shown that 2-(9-anthrylethyl) chloroformate (AEOC) is a sensitive and convenient pre-column derivatizing reagent for the determination of phenols by HPLC with fluorescence detection. AEOC-derivatized phenols have been shown to be very stable and the detection limits are similar to those found previously for AEOC-derivatized polyamines. The derivatization procedure is rapid, simple, and reproducible, making the method attractive for environmental analyses such as the determination of phenols in industrial waste waters and municipal water supplies.

Acknowledgements

Acknowledgement is made to the Camille and Henry Dreyfus Foundation Scholar/Fellow Program for providing major financial support for this research. The authors are also grateful to the National Science Foundation (REU grant, CHE9100-538) and the Dow Chemical Company Foundation and for providing summer research fellowships for K.J.H. and J.B.C. Finally, the authors express their gratitude to Professor Brian W. Williams, Bucknell University, for running fluorescence spectra and to Mr. Harry Wilson, Wilson Testing Laboratories, Shamokin, PA, USA, for providing industrial waste water samples.

References

[1] A.E. Greenberg, L.A. Clesceri and A.D. Eaton (Editors), Standard Methods for the Examination of Water and Wastewater, 18th ed., American Public Health Association, Washington, DC, USA, 1992.
[2] J. McMurry, Organic Chemistry, Brooks/Cole Publishing, Pacific Grove, CA, USA, 1992, pp. 1012–1013.
[3] J. Siegrist, C. Salles and P. Etievant, Chromatographia, 35 (1993) 50–54.
[4] T. Niwa, Clin. Chem., 39 (1993) 108–111.
[5] G. Montedoro, M. Servilli, M. Baldioli and E. Miniati, J. Agric. Food Chem., 40 (1992) 1571–1576.
[6] M. Akasbi, D.W. Shoeman and A.S. Csallany, J. Am. Oil Chem. Soc., 70 (1993) 367–370.
[7] G. Achilli, G.P. Cellerino and P.H. Gamache, J. Chromatogr., 632 (1993) 111–117.
[8] C. Li and M.W. Kemp, J. Chromatogr., 455 (1988) 241–251.
[9] K. Imai, T. Fukushima and H. Yokosu, Biomed. Chromatogr., 8 (1994) 107–113.
[10] M. Zheng, H. Xu and C. Fu, Sepu, 12 (1994) 186–188; Chem. Abstr., 121 (1994) 194612.
[11] C. Fu, Hebei Daxue Xuebao, Ziran Kexueban, 14 (1994) 35–40; Chem. Abstr., 121 (1994) 148045.
[12] S. Einarsson, B. Josefsson and S. Lagerkvist, J. Chromatogr., 282 (1983) 609–618.
[13] S. Einarsson, S. Folestad, B. Josefsson and S. Lagerkvist, Anal. Chem., 58 (1986) 1638–1643.
[14] S. Einarsson, B. Josefsson, P. Möller and D. Sanchez, Anal. Chem., 59 (1987) 1191–1195.
[15] J.R. Price, P.A. Metz and H. Veening, Chromatographia, 24 (1987) 795–799.
[16] A.J. Faulkner, H. Veening and H.-D. Becker, Anal. Chem., 63 (1991) 292–296.
[17] M.A. Cichy, D.L. Stegmeier, H. Veening and H.-D. Becker, J. Chromatogr., 613 (1993) 15–21.
[18] H. Sörensen, Ph.D. Thesis, University of Göteborg, Sweden, 1989.
[19] B.M. Mikhailov, Izv. Akad. Nauk SSSR Ser. Khim., (1948) 420–423; Chem. Abstr., 43 (1949) 208g.
[20] H.-D. Becker, L. Hansen and K. Andersson, J. Org. Chem., 51 (1986) 2956–2961.
[21] C.A. Dorschel, J.L. Ekmanis, J.E. Oberholtzer, F.V. Warren, Jr. and B.A. Bidlingmeyer, Anal. Chem., 61 (1989) 951A–968A.
[22] Test Method 604, GC Determination of Phenol, U.S. Environmental Protection Agency, Cincinnati, OH, USA, 1982.

ELSEVIER

Journal of Chromatography A, 718 (1995) 53–58

JOURNAL OF CHROMATOGRAPHY A

Differences in the glycosylation of recombinant and native human milk bile salt-stimulated lipase revealed by peptide mapping☆

Mats Strömqvist*, Kerstin Lindgren, Lennart Hansson, Kristina Juneblad

ASTRA HÄSSLE AB, Tvistevägen 48, S-907 36 Umeå, Sweden

First received 2 November 1994; revised manuscript received 25 May 1995; accepted 1 June 1995

Abstract

The milk of some mammals contains a bile salt-stimulated lipase (BSSL). Human milk BSSL is heavily glycosylated (30–40% carbohydrate) and present at a concentration of approximately 100–200 mg/l, thereby being one of the most abundant human whey proteins. BSSL has been shown to have an important role in the uptake of energy from human milk. The risk of HIV contamination has restricted the use of banked human milk for nutritional purposes. This has evoked an interest in the production of a recombinant form of the protein for supplementation of formula.

We have produced BSSL in mouse C127 and hamster CHO cells, and used chromatographic methods for the characterization of the products. This study was focused on study of the glycosylation of the protein by using peptide mapping and isolation of glycosylated fragments. The results show how human BSSLs from different sources differ both in extent of glycosylation, in glycan heterogeneity, and in lectin binding.

1. Introduction

Human milk contains a bile salt-stimulated lipase (BSSL) constituting approximately 0.5–1% of the total milk protein [1,2]. BSSL represents the major lipolytic activity in the milk and is therefore important for the uptake of energy from the milk [1]. The protein consists of 722 amino acids, carries one potential N-glycosylation site in the N-terminal part (Asn-187) and a repeat region of 16 segments of 11 amino acids each carrying several O-glycosylations close to the C-terminus (amino acids 536–711) [3,4]. By the study of mutants of the protein produced in C127 cells, non-glycosylated BSSL has been shown to have enzymatic activity similar to that of the glycosylated BSSL [5]. BSSL is sensitive to elevated temperatures and is inactivated by incubation at 40–50°C, thus pasteurization of the milk inactivates the enzyme [6]. As a major lipase in the human milk, BSSL might be interesting as a component of infant formula especially since pasteurization of donated human milk inactivates the enzymatic activity.

The aim of this study was to further characterize native and recombinant BSSL with emphasis on the glycosylation using high-performance liq-

* Corresponding author.

☆ Presented at the *14th International Symposium on the Separation and Analysis of Proteins, Peptides and Polynucleotides, Heidelberg, 2–4 November 1994.*

0021-9673/95/$09.50
SSDI 0021-9673(95)00632-X

uid chromatography. Additional information about the glycosylated repeat part of native as well as recombinant forms of BSSL produced in different cell lines could be obtained by purification of glycopeptides. Size-exclusion chromatography (SEC) gave information about the hydrodynamic size of the glycoproteins/glycopeptides and thereby the extent of glycosylation. Peptide mapping using reversed-phase liquid chromatography (RPLC) gave information on the heterogeneity of the glycan containing fragments. As a complement to these techniques, lectin affinity electroblotting was used to identify some of the glycan structures present in BSSL produced by the different cells.

2. Experimental

2.1. Purification of BSSL

Native BSSL

Human milk samples were collected from donors within the first three months of lactation. The donated milk was frozen until analyzed and purified. The milk was centrifuged at 30 000 *g* for 45 min. The fat layer and the pellet were removed and the remaining supernatant was filtered. To precipitate caseins, pH was adjusted to 4.3 with 1 *M* HCl and $CaCl_2$ was added to a final concentration of 60 m*M*. After incubation at room temperature for 1 h with stirring, the casein fraction was removed by centrifugation at 43 000 *g* for 90 min. The supernatant (whey) was dialyzed against 5 m*M* barbiturate-HCl, 50 m*M* NaCl, pH 7.4, applied to a Heparin-Sepharose column (Pharmacia Biotech., Uppsala, Sweden) and chromatographed as described previously [2]. Fractions were analyzed for presence of BSSL by ELISA and pooled for a second chromatography on Superdex 200 (Pharmacia Biotech.) equilibrated with 10 m*M* sodium phosphate, 0.5 *M* NaCl, pH 7.2.

Recombinant BSSL

Conditioned media harvested from roller-bottle cultures were filtered through a 0.22-μm sterile filter and thereafter chromatographed using the same two chromatographic steps as used for native BSSL. The purity, of both native and recombinant BSSL, was more than 95% according to densitometric scans of SDS-PAGE gels.

2.2. Enzymatic cleavage of BSSL

Carboxymethylation

Purified BSSL protein (5 mg) was dialyzed against distilled H_2O, lyophilized and dissolved in 1.5 ml 0.5 *M* ammonium acetate, 6 *M* guanidine-HCl. Dithiotreitol was added to a final concentration of 10 m*M* and the sample was incubated at room temperature under nitrogen for 1 h. Iodoacetic acid (0.5 *M*, freshly prepared) was added to a final concentration of 10 m*M* and the sample was incubated for another 2 h under nitrogen at room temperature in the dark. Finally the reaction was terminated by addition of 30 μl 2-mercaptoethanol and 525 μl glacial acetic acid.

Cleavage with trypsin

Carboxymethylated BSSL was dialyzed against 0.1 *M* NH_4HCO_3, pH 7.8. Trypsin [2% (w/w), sequencing grade, Boehringer-Mannheim Biochimica, Mannheim, Germany] was added and proteolytic cleavage was performed overnight (16–20 h) at 20–25°C. Digestions were conducted at a concentration of approximately 0.2–0.8 mg/ml of the carboxymethylated protein. The reaction was terminated by acidification to pH 3 with HCl and samples were stored on ice until analysis.

Cleavage with endoproteinase Lys-C

Carboxymethylated BSSL was dialyzed against 0.1 *M* NH_4HCO_3, pH 7.8 and cleaved with 1% (w/w) endoproteinase Lys-C (EndoLys-C, Boehringer-Mannheim) at 37°C. After 2 h of cleavage, another 1% EndoLys-C was added and the reaction was allowed to continue for 5 more hours. The reaction was conducted at approximately the same concentration as that with trypsin and was also terminated similarly. Total amino acid analysis was performed as described previously [7].

2.3. Chromatography

Chromatography was performed on a System Gold chromatographic system (Beckman Instruments, San Ramon, CA, USA) with a Model 126 pump and a Model 166 detector unit. Temperature control (for RPLC) was achieved by the use of a Waters TCM temperature control module (Millipore-Waters, Milford, MA, USA).

SEC

BSSL, digest as well as uncleaved protein, were applied on a TSK 3000 SW column (600 × 7 mm I.D., Beckman Instruments) equilibrated with 10 m*M* NaH_2PO_4, 0.3 *M* NaCl pH 7.2 at a flow-rate of 0.5 ml/min. As references for the molecular mass calculations, thyroglobulin (M_r 669), ferritin (M_r 440), IgG (M_r 150), bovine serum albumin (M_r 67) and ovalbumin (M_r 44) were used. Separations were carried out at 20°C.

RPLC

The protease digest was separated on a C_8 Ultrapore column (250 × 4.6 mm I.D., Beckman Instruments). The elution system consisted of two solvents: one consisting of 0.1% trifluoroacetic acid (TFA) in water and the second containing 90% acetonitrile and 0.089% TFA. The elution program was as follows: 0–5 min, 0% acetonitrile; 5–10 min, 0–12% acetonitrile; 10–55 min, 12–30% acetonitrile; 55–85 min, 30–60% acetonitrile. All gradients were linear, the flow-rate was 1 ml/min and the separations were made at 38°C.

2.4. Electroblotting and lectin staining

SDS-polyacrylamide gels were run according to Laemmli [8] using 4–15% precast polyacrylamide gels (Bio-Rad, Hercules, CA, USA). Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) using semi-dry electroblotting as described previously [9]. Membranes were blocked with 2% ovine serum albumin in 20 m*M* Tris-HCl, 0.5 *M* NaCl, pH 8.2 over-night at 4°C and washed twice in 50 m*M* Tris-HCl, 150 m*M* NaCl, pH 7.5 (TBS). The membranes were thereafter incubated for 1 h at room temperature with the different digoxigenin labeled lectins (Boehringer-Mannheim) diluted from the 1 mg/ml stock solutions with TBS containing 1 m*M* $MnCl_2$, 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$ as recommended by the manufacturer as follows:

Ricinus communis agglutinin (RCA)	1/100
Maackia amurensis agglutinin (MAA)	1/200
Aleuria aurantia agglutinin (AAA)	1/1000
Phytohemagglutinin-L (PHA-L)	1/200
Sambucus nigra agglutinin (SNA)	1/1000
Peanut agglutinin (PNA)	1/100
Concanavalin A (Con A)	1/100
Galantus nivalis agglutinin (GNA)	1/1000
Wheat germ agglutinin (WGA)	1/100
Datura stramonium agglutinin (DSA)	1/1000

After lectin incubations, membranes were washed 3 × 10 min in TBS and incubated 1 h at room temperature with alkaline phosphatase conjugated anti-digoxigenin Fab fragments (Boehringer-Mannheim) diluted 1/1000 in TBS. Membranes were washed 3 × 10 min in TBS and bound enzyme activity was detected as described [9].

3. Results and discussion

Cleavage of BSSL, enzymatically or chemically, results in a rather strange distribution of fragment sizes. Four different cleavage methods were tried; chemical cleavage with CNBr and enzymatic cleavages with endoproteinase Lys-C (EndoLys-C), trypsin and endoproteinase Glu-C (EndoGlu-C). Enzymatic cleavage with Endo-Glu-C was not successful while the other three methods worked better. Cleavage with CNBr, trypsin or EndoLys-C resulted in the liberation of the repetitive sequence domain of 16 × 11 amino acids contained within a single fragment of approximately 200 residues. Cleavage of BSSL with EndoLys-C gives theoretically 38 peptide fragments (Fig. 1), 12 of which having only three or less amino acids and one being as long as 201 residues (EndoLys-C 37, amino acids 512–712 containing the 16 × 11 repeat region).

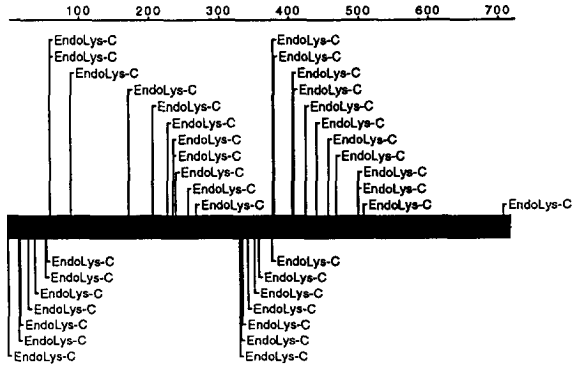

Fig. 1. Cleavage of BSSL with EndoLys-C. The positions of the cleavage sites are indicated by the vertical bars. The upper numbered horizontal bar represents the polypeptide backbone from N- to C-terminus.

The peptide maps obtained on reversed-phase chromatography of the three different forms of BSSL showed reasonably well separated peptides except for the peaks eluting at approximately 50–55 min (Fig. 2). The amino acid analysis of collected material in this region showed that the fragment containing all the repeats was present in these fractions. The origin of most of the other fragments could also be determined by amino acid analysis as indicated in Fig. 2. When uncleaved BSSL (native as well as recombinant) was analyzed by size-exclusion chromatography, the elution behavior of the protein indicated a large hydrodynamic molecular size of the protein ($>M_r$ 300, Table 1). Due to the presence of some arginines prior to the repeat part of this fragment, enzymatic cleavage with trypsin yields a somewhat shorter and more absolute repeat fragment (amino acids 521–712). For this reason, and also because trypsin is an economically better choice when cleavage is to be done for preparative purposes, trypsin instead of Endo-Lys-C was chosen for the further study of the O-glycosylation. Analysis of the tryptic digests of the proteins variants by SEC shows that the reason for the behavior as a very large protein on SEC resides in one fragment in the digest eluting early in the chromatogram (O-GF, Fig. 3). We could confirm that this fragment was the repeat fragment carrying the O-glycosylations. This fragment accounts for a molecular mass of approximately 18 when not considering the glycosylations. The elution position of the fragment in size-exclusion chromatography indicates a molecular mass above 200 (Fig. 3, Table 1). Thus, the behavior of the protein as being very large appears to be due to a large hydrodynamic size of this glycosylated fragment. This fragment could easily be prepared just by cleaving BSSL with trypsin. SEC could be used as a simple tool for comparison of the size of the fragment and thereby giving a hint on the extent of O-glycosylation of recombinant and native BSSL. Interestingly, the sizes obtained from SEC were very close when comparing the native

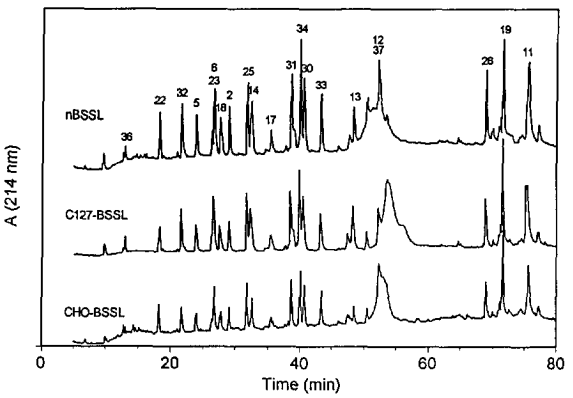

Fig. 2. Peptide map obtained on RPLC of the BSSL variants obtained after cleavage with EndoLys-C. The protein was cleaved as described under Experimental and applied to the column. In each run, 100 μl sample at a concentration close to 1 mg/ml was loaded on the column. Conditions for the chromatography are given in the Experimental section. The numbers represent the fragment number from N- to C-terminus. Abbreviations: nBSSL = native BSSL, C127-BSSL = BSSL produced in C127 cells, CHO-BSSL = BSSL produced in CHO cells.

Table 1
Molecular masses of the intact BSSL variants and their O-glycosylated repeat fragments obtained by SEC

Type of BSSL	M_r intact protein	M_r glycopeptide
Native BSSL	321 ± 6	259 ± 5
C127-BSSL	331 ± 12	255 ± 7
CHO-BSSL	336 ± 7	260 ± 3

The figures represent the mean of three chromatographies.