SHORT COMMUNICATION

Thermostable trypsin conjugates for high-throughput proteomics: synthesis and performance evaluation

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Conjugating bovine trypsin with oligosaccharides maltotriose, raffinose and stachyose increased its thermostability and suppressed autolysis, without affecting its cleavage specificity. These conjugates accelerated the digestion of protein substrates both in solution and in gel, compared to commonly used unmodified and methylated trypsins.

Received: August 1, 2005
Revised: December 30, 2005
Accepted: January 9, 2006

Keywords:
Bovine trypsin / Conjugate / In-gel digestion / Oligosaccharide / PMF

In a typical proteomic routine, isolated proteins are digested by trypsin in solution or in gel, and the yielded peptides are further characterized by MS [1]. Under typical digestion conditions (pH ~8), trypsin (EC 3.4.21.4) exclusively cleaves peptide bonds C-terminal of arginine and lysine residues, if they are not followed by a proline residue [2]. Peptides with internal arginine and lysine residues are relatively rare. The average size of tryptic peptides (10–12 amino acids) and C-terminal location of basic amino acid residues make them readily amenable to MS analysis by both MALDI and ESI techniques, including *de novo* sequencing [3, 4].

Two major drawbacks hamper the application of trypsin in proteomics. The enzyme shows only marginal thermostability at 37°C and undergoes rapid autolysis under basic pH, even in the presence of stabilizing calcium ions [5]. In this context, trypsin with methylated lysine residues showed better thermostability and autolysis resistance, which enabled faster cleavage of protein substrates at elevated temperature and higher enzyme concentration, without compromising the yield of digestion products [6, 7]. However, methylation did not eliminate trypsin autolysis completely, and this set an upper practical limit for its concentration in the digestion buffer.

Considerable effort has been dedicated to enhancing trypsin stability by conjugation with water-soluble polymers. Villalonga *et al.* [8] for example prepared an active conjugate with carboxymethylcellulose, which showed significantly increased thermal and pH stability. The conjugate was also more resistant to the action of denaturing agents. We therefore reasoned that trypsin conjugation with oligosaccharides might yield an autolysis resistant, thermostable and hydrophilic form of the enzyme. At the same time, since oligosaccharide moieties are relatively small, both the size of conjugate molecules and their in-gel diffusion properties would not be much affected.

Bovine trypsin (BT) used for conjugate synthesis was from ICN Biomedicals (Aurora, OH, USA; cat. no. 101179). Methylated porcine trypsin (MET-PT) was from Promega (Mannheim, Germany). Other chemicals, including all oli-

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Abbreviations: ACD, α-cyclodextrin; **BAPNA**, N^{α}-benzoyl-DL-arginine 4-nitroanilide; **BCD**, β-cyclodextrin; **BT**, bovine trypsin; **LAC**, lactose; **MAL**, maltose; **MAT**, maltotriose; **MEL**, melibiose; **MET-PT**, methylated porcine trypsin; **RAF**, raffinose; **RAFR**, RAF plus biacetyl; **STA**, stachyose

gosaccharides, were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Lactose-, maltose- and melibiose-modified BT (LAC-BT, MAL-BT, MEL-BT, respectively) were prepared as described in [9] with minor modifications. BT (75 mg) was dissolved in 10 mL PBS pH 7.5, containing 10 mM benzamidine. After adding the disaccharide and sodium cyanoborohydride, the reaction mixture was stirred for 30 min and then incubated at 30°C for 48 h. Maltotriose-, raffinose-, stachyose-, α-cyclodextrin- and β-cyclodextrin-modified BT (MAT-BT, RAF-BT, STA-BT, ACD-BT and BCD-BT, respectively) were synthesized following a described conjugation protocol [10]. An aliquot of RAF-BT was further treated with biacetyl yielding an RAFR-BT conjugate with modified arginine residues [11]. Crude conjugates were dialyzed against 20 mM sodium acetate buffer pH 4.0, and then purified by ion exchange chromatography on a Mono S HR 5/5 column (Amersham Biosciences) using a gradient 0–1 M NaCl in the dialyzing buffer. The collected conjugates were dialyzed against 0.1% formic acid, concentrated by ultrafiltration (a 10-kDa cut-off filter), lyophilized and stored at −80°C.

Trypsin activity was determined using a chromogenic substrate N^{α}-benzoyl-DL-arginine 4-nitroanilide (BAPNA) at an initial concentration of 1.25 mM as described [7]. Thermostability of BT and its conjugates was evaluated by monitoring the changes in their specific activity upon incubating enzyme aliquots in 20 mM sodium acetate buffer pH 4.0 at 20°, 37°, 45°, 55°, 65° and 75°C for 30 min, followed by rapid cooling in water-ice bath. Protein content was determined using bicinchoninic acid [12]; RAF-BT, RAFR-BT and BCD-BT were also subjected to amino acid analysis in the laboratory of Dr. P. Hunziker (University of Zürich, Switzerland). Total carbohydrates were determined by the phenol-sulfuric acid method [13]. Primary amino groups were determined using 2,4,6-trinitrobenzenesulfonic acid [14]. IEF was performed on a vertical slab gel [15] with Servalyte 2-11 (Serva, Heidelberg, Germany). The molecular mass of BT and its conjugates was estimated by tricine-SDS-PAGE [16] or, where specified, by MALDI-TOF MS. In the latter case, 1 μL of the conjugate (10 mg/mL in 0.1% formic acid) was mixed with 3 μL of "super" dihydroxybenzoic acid (sDHB from Bruker, 10 mg/mL in 1% TFA:ACN, 1:1). Aliquots (0.6 μL) were placed onto the target plate and allowed to dry. Spectra were acquired in the linear mode for positive ions and calibrated externally.

Eight conjugates were synthesized by coupling oligosaccharides to lysine residues of BT. To obtain LAC-BT, MAL-BT and MEL-BT, the enzyme was reacted directly by the aldehyde (acyclic) forms of the disaccharides in presence of sodium cyanoborohydride, which reduced intermediate Schiff bases. MAT-BT, RAF-BT, STA-BT, ACD-BT and BCD-BT were synthesized by the reaction of BT with oligosaccharides (two trisaccharides, a tetrasaccharide and cyclodextrins, respectively) activated by potassium periodate oxidation. This was followed by reduction with cyanoborohydride as above. In both methods, BT was protected from autolysis during the reaction by its competitive inhibitor benzamidine. An aliquot of RAF-BT was additionally modified by biacetyl to acylate arginine residues, which was supposed to bring further stabilization against autolysis.

The extent of trypsin modification by oligosaccharides was independently determined in three ways: by spectrophotometric quantification of free amino groups, by spectrophotometric determination of carbohydrate content, and, in some cases, by the number of unmodified lysine residues determined by amino acid analysis. Considering the total number of lysine residues in its sequence plus the N terminus, BT comprises 15 primary amino groups [8]. Despite large molar excess of the modifying reagents used, a maximum of 9 modified lysine residues was achieved. The carbohydrate content of the synthesized conjugates was in the range of 8–25%, which agreed well with the corresponding number of modified lysine residues. The amino acid analyses of RAF-BT, RAFR-BT and BCD-BT confirmed that the content of free lysine residues was substantially decreased (5, 4 and 5 residues per molecule, respectively).

The molecular masses of the conjugates were determined by discontinuous tricine-SDS-PAGE and by MALDI-TOF MS. As anticipated, the molecular mass of the disaccharide conjugates of BT (~25 kDa) was comparable with that of native BT (~23 kDa), whereas for the other conjugates it was significantly higher (~27–33 kDa). The molecular mass of RAF-BT conjugate was directly determined by MALDI-TOF MS as 26.29 kDa (Fig. 1A). Similarly, MAL-BT and STA-BT masses were determined as 25.23 kDa and 28.52 kDa, respectively. Intact BT was detected as a narrow symmetric peak corresponding to a more accurate mass value of 23.292 kDa. After the coupling with relatively large molecules of the activated cyclodextrins, a strong mass heterogeneity of the produced BT conjugate was apparent. For example, MALDI-TOF mass spectrum of BCD-BT revealed a series of partially resolved peaks between *m/z* 29 032 and 33 260 with the most abundant component at *m/z* 31 126. The mass differences between adjacent peaks in the series matched the mass of β-cyclodextrin (not shown). The single-chain form of BT (β-trypsin) is a strongly basic protein with p*I* 10.5 [17]. Since lysine residues significantly contribute to the net charge, IEF was performed to estimate how their modification affected the enzyme p*I*. The RAF-BT band in IEF gel was more acidic (p*I* 6.7), compared to native BT. Similar p*I* values were determined for MAT-BT (6.6), RAFR-BT (5.9), STA-BT (6.3) and BCD-BT (6.1).

To characterize kinetic properties of the synthesized conjugates, we determined their specific activity and K_m using a low-molecular-mass substrate BAPNA. The modification decreased the specific activity by 10–30% compared to that of unmodified BT (28 nkat/mg), most substantially for the disaccharide conjugates. Their K_m were all in the millimolar range, with no considerable difference compared to unmodified BT (K_m = 2.8 mM). To estimate the thermostability of the conjugates, we determined their T_{50} constants, defined here as a temperature at which 50% of the activity is retained upon 30-min incubation. T_{50} of BT was

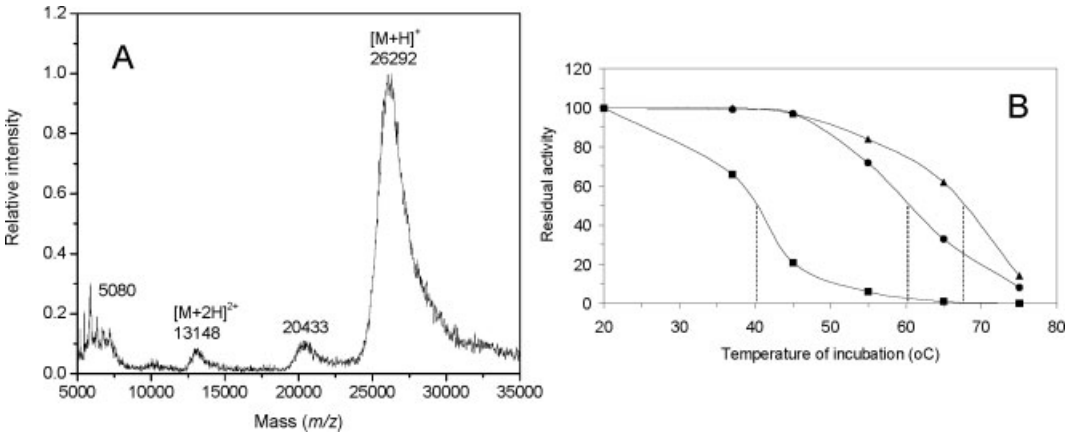

Figure 1. Properties of BT conjugates with oligosaccharides. (A) MALDI-TOF mass spectrum of intact RAF-BT acquired in the linear mode. (B) Characterization of thermostability of BT conjugates. Enzyme aliquots were incubated at different temperatures ranging from 20° to 75°C for 30 min: BT (-■-); MAT-BT (-●-), BCD-BT (-▲-). After rapid cooling, residual activity was determined by hydrolysis of BAPNA substrate at 30°C. The corresponding T_{50} values are indicated by vertical lines.

41°C. For LAC-BT, MAT-BT and MEL-BT, it was higher by ~10°C, and for MAT-BT, RAF-BT, RAFR-BT and STA-BT by ~20°C. Among all conjugates, ACD-BT and BCD-BT were the most stable, with T_{50} values close to 70°C (Fig. 1B). Importantly, the higher T_{50} was reflected in accelerated cleavage of BAPNA at elevated temperatures. The reaction rate of RAF-BT-catalyzed reaction increased up to 55°C and then did not change up to 70°C, whereas for BT it rapidly declined above 50°C (data not shown).

To evaluate the performance of the trypsin conjugates in in-gel proteolysis, samples of standard proteins (5 pmol) were separated on a 12% polyacrylamide gel and visualized by CBB. The proteins were in-gel digested following a modified accelerated protocol [7], and then peptide mass fingerprints were acquired from 1-μL aliquots withdrawn directly from the digest. MALDI-TOF MS was performed on a Reflex IV instrument (Bruker Daltonik GmbH, Bremen, Germany) as described previously [7]. The conjugate concentration in 50 mM ammonium bicarbonate digestion buffer was 1.0 μM and the cleavage process was carried out at 55°C for 3 h. LAC-BT, MAL-BT and MEL-BT provided low peptide yields in pilot experiments. ACD-BT and BCD-BT, which efficiently digested proteins in solution, demonstrated only marginal activity in in-gel digestion. Therefore only MAT-BT, RAF-BT, RAFR-BT and STA-BT were subjected to further tests. The reaction temperature (55°C) was selected to balance the reaction rate against the rate of thermal inactivation, both of which accelerate along with the temperature increase. The relatively high load of protein standards allowed us to acquire spectra that were rich in tryptic peptides, and hence we could better evaluate and pinpoint possible changes in the cleavage specificity of the trypsin conjugates. In acquired MALDI-TOF spectra, m/z of all peaks with sn S/N ratio >2 were fetched and used for searches against MSDB protein sequence database (May 15, 2005); mass tolerance was 150 ppm. We then compared the sequence coverage (determined as % of the full-length protein sequence covered with the matched peptides) of peptide mass maps of the digests produced by the BT conjugates with the maps obtained by conventional digestion using BT (37°C, overnight) or accelerated digestion using MET-PT (3 h at 55°C) [7] (Table 1).

To determine the number and relative abundance of autolysis products of the conjugates, we performed control digests of blank gel slabs of a similar size (1.0 μM BT or trypsin conjugates in 50 mM ammonium bicarbonate digested overnight at 37°C). MALDI-TOF mass spectra of autodigests of MAT-BT, RAF-BT, RAFR-BT and STA-BT were acquired and compared with the autolytic peptide pattern of BT. Major peaks of autolysis products of BT were detected at m/z 1020.54, 2163.06, 2193.03, 2273.18, 2289.18 and 2305.10 (Fig. 2A). However, only three of them (at m/z 2163.1, 2273.2 and 2289.2) were also found in the autodigests of MAT-BT, RAF-BT, RAFR-BT and STA-BT (Fig. 2B). The intensity ratio of the major peaks (m/z 2163.1 and 2273.2) strongly varied among the spectra. Importantly, the autodigests of the conjugates contained fewer minor autolytic peptides. Thus, the specificity of protein identification was enhanced (Fig. 2B).

The kinetics of in-gel digestion of proteins is affected by limited diffusion of proteases into the matrix of polyacrylamide gels [7, 18]. Therefore, for conjugating with BT, we selected oligosaccharides such that, if lysine residues are almost completely modified, the molecular mass of the conjugate would not exceed 35 kDa. The determined molecular masses of the synthesized conjugates (~25–33 kDa) were generally in a good agreement with the number of modified lysine residues, carbohydrate content and apparent p*I*.

Table 1. MALDI TOF peptide mass fingerprints of protein standards in-gel digested by BT and its conjugates

Protein standard[a)]	BT (Roche)		MET-PT (Promega)		MAT-BT		RAF-BT[b)]		RAFR-BT		STA-BT	
	Peptides	Coverage (%)	Peptides	Coverage (%)	Peptides	Coverage (%)	Peptides	Coverage (%)	Peptides	Coverage (%)	Peptides	Coverage (%)
Cytochrome c (CCHO)	7	53	10	63	7	30	5	45	6	45	7	43
Myoglobin (MYHO)	12	76	10	71	11	74	11	74	11	74	12	80
Aldolase (ADRBA)	15	34	19	49	15	46	15	39	17	45	17	47
BSA (AAA51411)	20	32	21	35	16	27	19	28	14	20	10	17

a) Codes in parentheses represent the respective Pcodes in the MSDB database.

b) Additional results with RAF-BT: glucose-6-phosphate dehydrogenase from baker's yeast (MSDB Pcode G6PD_YEAST, 10 peptides, coverage 21%), recombinant maize cytokinin oxidase 1 (MSDB Pcode T51929, 22 peptides, coverage 36%), recombinant *Arabidopsis thaliana* cytokinin oxidase 2 (MSDB Pcode AAO42130, 23 peptides, coverage 45%), pea seedling amine oxidase (MSDB Pcode JC7251, 21 peptides, coverage 37%) and rabbit glycogen phosphorylase chain B (MSDB Pcode 1GPB, 6 peptides, coverage 19%).

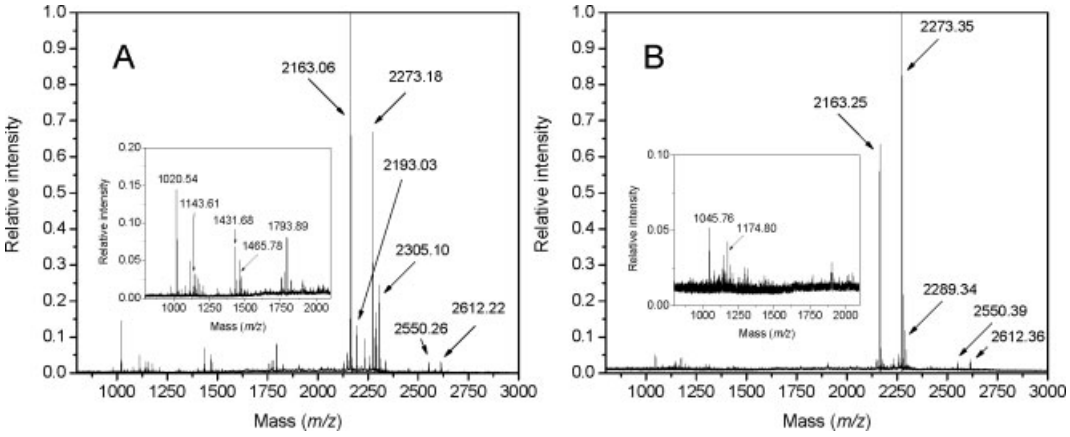

Figure 2. Peptide mass fingerprints of autolysis products of BT and MAT-BT. Unmodified BT and its conjugate with MAT-BT (both 1.0 μM) were incubated in 50 mM ammonium bicarbonate at 37°C for 12 h. Then aliquots (1 μL) were withdrawn and analyzed by MALDI-TOF MS using a CHCA matrix. (A) Peptide mass fingerprint of BT autolyzate; (B) peptide mass fingerprint of MAT-BT autolyzate.

Although oligosaccharide conjugating has been introduced as a facile and inexpensive method [19], the reaction with BT was rather incomplete (in agreement with previous results [8]) and yielded a mixture of molecular forms with variable modification degree as demonstrated by MALDI-TOF MS. The determined K_m values of the BT conjugates agreed well with those previously reported for BT and BT modified by small succinyl and guanyl moieties [20], suggesting that the conjugation did not affect substrate binding at the active site. At the same time, the modification significantly enhanced thermostability as compared to unmodified BT. Interestingly, the larger size of oligosaccharide modifiers increased T_{50}, although no noticeable increase in the completeness of the modification was observed.

We further demonstrated that MAT-BT, RAF-BT, RAFR-BT and STA-BT performed well under typical conditions of the accelerated in-gel digestion of proteins [7]. Molecular masses of these conjugates did not exceed 27 kDa which enabled facile substrate cleavage within the matrix of 12% polyacrylamide gel having an apparent pore size of 20–30 Å [21]. It was therefore not surprising that ACD-BT and BCD-BT were not active in in-gel digestion, although they readily cleaved both BAPNA and protein substrates in solution. The sequence coverage and MOWSE scores of the peptide mass maps acquired from the digests with conjugates or MET-PT (3 h at 55°C) and with BT (overnight at 37°C) were similar, although quantitative kinetic measurements have yet to be performed. Because of the increased thermostability, in-gel digestion could be performed at elevated temperature (*e.g.*, 55°C), which promotes denaturing of protein substrates without chaotropic agents or detergents. This might have important implications for in-solution digestion of complex protein mixtures.

The studied BT conjugates produced less autolysis products in the *m/z* range of 700–2700 compared to BT, and therefore low abundant peptides from the target protein were easier to recog-

nize. However, the most abundant peaks of autolysis products of BT (*m/z* 2163 and 2273) were also detected in autodigests of the conjugates. Interestingly, these peptides both contain a C-terminal lysine residue, but not an arginine residue. The sequence of bovine trypsinogen (NCBI protein accession code P00760) comprises 243 amino acids, from which the region 21–243 represents β-trypsin. The peptides mentioned above are located in the successive positions 70–89 and 90–109. The crystal structure of BT complex with 2-aminobenzimidazole was downloaded from the RCSB Protein Data Bank (www.rcsb.org/pdb; accession code 1QA0). Computer structure examination using the DeepView/Swiss-PdbViewer v3.7 software (www.expasy.org/spdbv) revealed that Lys89 and Lys109 are located at the molecule surface and are spaced by 7 Å. Because of sterical constraints, they probably cannot both react with bulky modifiers (for example, the size of ACD molecule is about 20 Å [22]), and for that reason only one group per molecule was modified.

Taken together, our data suggest that MAT-BT, RAF-BT, RAFR-BT and STA-BT are perspective enzymes for gel-based and gel-free proteomic applications. We have demonstrated that trypsin modification by oligosaccharides preserved its valuable cleavage specificity, yet increased the thermal stability, optimal digestion temperature and reduced autolysis.

This work was supported by the grants KONTAKT ME664, MSM 6198959216 and MSM 0021622415 from the Ministry of Education, Youth and Sports, Czech Republic and by the joint project KONTAKT-mobilita CZE 02/003 to M.S. and A.S. within a bilateral scientific cooperation (granted by both the Czech Ministry of Education, Youth and Sports and the German Bundesministerium für Bildung und Forschung).

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