Porcine Gastric TFF2 is a Mucus Constituent and Differs from Pancreatic TFF2

René Stürmer a, Stefan Müller b, Franz-Georg Hanisch b, Werner Hoffmann a

aInstitute of Molecular Biology and Medicinal Chemistry, Otto-von-Guericke University Magdeburg, Magdeburg, bInstitute of Biochemistry II, Medical Faculty, and Central Bioanalytics, Center for Molecular Medicine Cologne, University Köln, Köln, Germany

Key Words
Mucins • Mucus • Pancreas • Stomach • Peptide interactions • TFF peptide • Trefoil factor • Spasmolytic polypeptide

Abstract
Background/Aims: TFF2 is a secretory peptide (106 amino acid residues) of the gastric mucosa, the porcine exocrine pancreas as well as immune cells and the CNS. It was the aim of this study to compare gastric and pancreatic TFF2. Methods: TFF2 was purified from the porcine stomach and pancreas, respectively, by size-exclusion and anion-exchange chromatography and then analyzed by Western blots as well as mass spectrometry. Results: Gastric and pancreatic TFF2 differed markedly, i.e. gastric TFF2 was exclusively associated with the high molecular mass mucus fraction, whereas pancreatic TFF2 appeared as a low molecular mass product. Unexpectedly, the latter predominantly formed a non-covalently linked homodimer resistant to boiling SDS. In contrast, gastric TFF2 is an integral mucus constituent predominantly binding to the mucin MUC6 in a non-covalent fashion. Conclusion: The non-covalent interaction of TFF2 with the mucin MUC6 is typical of a "link peptide" which is perfectly suited to assemble and stabilize the laminated structure of gastric mucus and to modulate its rheological properties.

Introduction

TFF2 (originally termed "pancreatic spasmolytic polypeptide") is a secretory peptide of the trefoil factor family (TFF) consisting of 106 amino acid residues [reviews: refs. 1, 2]. It contains two TFF domains, which are stabilized by three highly conserved disulfide bridges each and a seventh disulfide bridge between Cys-6 and Cys-104; the latter is responsible for a compact circular structure by closing the N- and C-termini [3-5]. In solution it has been described as a monomer [4, 6]. TFF2 is an exocrine secretory product of the porcine pancreatic acinar cells as well as the gastric mucosa and Brunner’s glands [7]. In human, the major site of expression is the stomach [8] where TFF2 is secreted together with the mucin MUC6.
from cardiac glands, mucous neck cells, and antral gland cells [9, 10]. There is a dramatic diurnal variation of TFF2 in the human gastric juice [11] and TFF2 is an early response gene after gastric mucosal injury [12]. Pathologically, TFF2 is expressed in the ulcer-associated cell lineage during inflammatory conditions [13] as well as in the metaplastic SPEM lineage [14]. Human TFF2 is N-glycosylated containing a fucosylated N,N'-diacetylchitosaminoligosaccharide [15]; whereas porcine, murine, and rat TFF2 are devoid of N-glycosylation.

TFF2 is a typical constituent of the layered gastric mucus. In the rat, mouse, and pig two-layered structures have been reported [16-18]; whereas in human the gastric mucus is multi-layered [19]. In the latter, TFF2 is specifically localized within the alternating array of two types of mucins; TFF2 is co-localized with the gland mucin MUC6 and not with the surface mucous cell mucin MUC5AC indicating a physical interaction of TFF2 and MUC6 [10, 20, 21]. Of special note, Helicobacter pylori preferentially colonizes the MUC5AC gel layer [22] probably because the terminal O-glycans of MUC6 have antimicrobial activity against H. pylori [23]. A physical interaction of TFF2 and high molecular mass constituents of mucus (such as mucins) was also observed after size exclusion chromatography (SEC) [15, 24]. Furthermore, TFF2, and not TFF1 or TFF3, increased dramatically the viscosity and elasticity of porcine gastric mucin solutions in vitro [25]. Similar results were obtained with systemically administered TFF2 in vivo [26]. TFF2-mucin solutions showed non-Newtonian pseudo-plastic behaviour typical of an entangled network [25]. The viscous response was pH-dependent with highest viscosity at low pH indicating hydrogen bonding [25].

Thus, a physical interaction of TFF2 with mucin constituents may strengthen the mucus barrier and would explain why only orally administered TFF2 and not parenteral TFF2 protected against induced gastric injury as well as induced colitis in rats [27, 28]. In line with this hypothesis, TFF2 applied luminally to the gastric mucosa inhibited H+ permeation through the mucus barrier [29] and Tff2-deficient mice (Tff2−/−) have induced susceptibility to Helicobacter-induced gastritis [30], they showed accelerated progression to dysplasia [31], and their gastric surface pH was attenuated after photodamage [32]. Thus far, the interaction partners of TFF2 and the type of interaction are not known.

TFF2 is also expressed in the brain and pituitary [33] as well as in immune cells such as macrophages and lymphocytes [30, 34]. Tff2−/− mice show an altered expression of genes implicated in immune regulation [35] and their immune cells are hyperresponsive to IL-1β stimulation [30].

Also relatively weak motogenic effects have been observed for TFF2 in vitro [compilation: 36]; this activity is dependent on the ERK1/2 pathway [37, 38]. Furthermore, TFF2 is considered as a low affinity ligand for the chemokine receptor CXCR4 [39, 40]. However, at present there is a lack of a clear receptor for TFF2. In the past, integrin β1 as well as a large membrane glycoprotein with similarity to CRP-Ductin/DMBT1 have been identified as TFF2 binding proteins in the porcine stomach [41]. Here, we systematically compare TFF2 from porcine stomach and pancreas, respectively.

**Materials and Methods**

**Extraction of proteins from porcine stomach or pancreas**

Porcine stomach (antral region) and pancreas were obtained from a local abattoir. Principles of animal care and specific national laws were followed. Extraction of the gastric or pancreatic specimens with a 5-fold amount (w/v) of buffer was performed as described previously in detail [15].

**Protein purification by FPLC**

The gastric or pancreatic extracts (about 10 mL each) were fractioned by SEC with the ÄKTA™ - FPLC system (Amersham Biosciences, Freiburg, Germany) as described previously [15]. The following columns were used (from GE Healthcare Bio-Sciences AB, Uppsala, Sweden): HiPrep 16/60 Sephacryl S-300 High Resolution (flow rate: 0.5 mL/min, 1.8 mL fractions) or HiLoad 16/600 Superdex 75 prep grade (flow rate: 1.0 mL/min, 2.0 mL fractions). Anion-exchange chromatography using a Resource Q6 column (Amersham Biosciences; flow rate: 6.0 mL/min, 1.0 mL fractions) was performed as described previously [42].
SDS-PAGE, SDS-AgGE, and Western blot analysis

Denaturing SDS-PAGE under reducing (5% β-mercaptoethanol) or non-reducing conditions, non-denaturing SDS-agarose gel electrophoresis (AgGE) under non-reducing conditions, protein staining with Bio-Safe Coomassie Stain (Bio-Rad Laboratories GmbH, Munich, Germany), silver staining, and periodic acid-Schiff (PAS) staining for mucins (dot blot) have been previously described [42]. For special non-reducing SDS-PAGE the concentration of the stacking gel was lowered to 5%; this enables also detection of very high molecular mass products. Gels obtained after non-reducing SDS-PAGE were subsequently reduced with 1% β-mercaptoethanol at 50°C for 5 min (post-in-gel reduction).

Western blot analysis after SDS-PAGE, electrophoretic transfer, and fixation with 0.2% glutaraldehyde were performed according to previous reports [24, 43]. Western blot analysis after SDS-AgGE has been described previously [42] using either capillary blotting or electrophoretic transfer. The porcine mucin MUC6 was detected with the biotinylated lectin GSA-II (from *Griffonia simplicifolia II*; Biozol Diagnostica Vertrieb GmbH, Eching, Germany) [44] followed by binding of a streptavidin-peroxidase conjugate (Roche Diagnostics GmbH, Mannheim, Germany). The porcine mucin MUC5AC was detected with the polyclonal antiserum PGM2B-1 [44; kindly provided by Prof. J. R. Davies, Malmö]. TFF2 was detected with the affinity-purified polyclonal antiserum anti-hTFF2-1 [45]. Bands were visualized with the ECL detection system and semi-quantitative analysis of the bands was performed using the GeneTools software (version 3.07; Syngene, Cambridge, UK) as described in detail [24].

LC-ESI-MS/MS analysis of in-gel digested proteins, database searching

Tryptic digestion of Coomassie stained protein bands and LC-MS/MS analysis have been described in detail previously [42]. Additionally, portions of the Coomassie stained protein band were also digested with chymotrypsin or V8 protease. Trypsin and chymotrypsin digestion were performed in 50 mM ammonium bicarbonate buffer, while 100 mM Tris/HCl pH 8.0 was used with the V8 protease. Extracted peptides of all digests were combined and analyzed in a single run.

Proteins were identified by searching the NCBI nr database (NCBI nr 20130730; 31350673 sequences; 10834990394 residues). Searches were submitted to MASCOT 2.4.0 (Matrix Science Ltd., London, UK) by Proteinscape 3.0 (Bruker Daltoniks, Bremen, Germany) with the enzyme parameter setting "none". Additional setting were: species "*Sus scrofa*", fixed modifications "carbamidomethyl", optional modifications "methionine oxidation" and "Gln->pyro-Glu". The mass tolerance was set to 0.3 Da for peptide and fragment spectra.

MALDI-TOF/TOF mass spectrometry

Briefly, a FPLC fraction containing purified TFF2 was passed through a C4 ZipTip (Millipore) equilibrated in 0.1% TFA. The tip was washed three times with 10 µL 0.1% TFA. TFF2 was eluted with 10 µL 70% acetonitrile in 0.1% TFA and mixed 1:1 with a saturated solution of sinapinic acid in 50% acetonitrile, 0.1% TFA. Protein calibration standard I containing insulin, ubiquitin cytochrome C, and myoglobin (Bruker Daltonics, Bremen, Germany) in 0.1% TFA was mixed 1:1 with the saturated sinapinic and an equal volume was added to the sample. 1 µL of this mixture was applied onto a stainless steel target and linear MALDI mass spectrometry was performed on an UltraflexTreme MALDI TOF instrument in positive mode (Bruker Daltonics, Bremen, Germany). Singly charged ions corresponding to the standard proteins were used for internal calibration of the mass range from m/z 5734 to m/z 16952.

Results

Characterization of gastric and pancreatic TFF2 by SEC and Western blot analysis

Extracts of porcine stomach or pancreas were subject to SEC (Fig. 1). In gastric extracts, the major amount of TFF2 immunoreactive material was associated with the PAS-positive high molecular mass fraction (fractions 20-24; Fig. 1A). In contrast, TFF2 from pancreatic extracts was eluted with low molecular mass fractions (fractions 51-55; Fig. 1B).

SDS-PAGE under non-reducing conditions and subsequent Western blot analysis of the TFF2-enriched fractions from porcine stomach or pancreas revealed different patterns (Fig.
1D). Typical of gastric extracts is TFF2 immunoreactive material in the very high molecular mass region, which hardly enters the stacking gel. In contrast, pancreatic extracts showed a predominant TFF2 immunoreactive band with a \( M_r \) of about \( \leq 30 \text{k} \). Furthermore, both gastric and pancreatic extracts contained a TFF2 immunoreactive 16k-band, which probably represents TFF2 monomers. After reduction, only monomeric TFF2 was observed in both gastric and pancreatic extracts (Fig. 1C).

**Characterization of TFF2 in the porcine gastric mucus complex**

In order to further analyze the high molecular mass complex of gastric TFF2, an extract of mucus scrapings from the antral porcine mucosa was separated by SEC (Fig. 2A). The major amount of TFF2 immunoreactivity was roughly associated with the PAS-positive high molecular mass fractions (Fig. 2A). Then, the TFF2-positive peak material (fractions 19-21) was subsequently purified by anion-exchange chromatography (Fig. 2B). Of note, the TFF2-positive material appeared as a broad peak (fractions 17-41) partly congruent with PAS-positive material. These fractions were subsequently separated by SDS-AgGE followed by Western blot analysis for TFF2, MUC5AC, and GSA-II (Fig. 2C). Heterogeneous MUC5AC was mainly found in fractions 18-22; whereas TFF2 was detectable in fractions 16-24 with a \( M_r \) even higher than that of MUC5AC, hardly entering the gel. A similar pattern was obtained for GSA-II, which recognizes porcine MUC6 [44]. This result is outlined in Fig. 2D by directly comparing Western blot analyses for TFF2, MUC5AC, and GSA-II on the same gel.
Purification of TFF2 from the porcine pancreas

Another aim of this study was to purify and identify the novel ≤30 k form of pancreatic TFF2. Thus, the pancreatic extract was separated by SEC (Fig. 3A) and the TFF2 positive fractions were subsequently purified by anion-exchange chromatography (Fig. 3B). As a last step, TFF2 positive fractions from the anion-exchange column were again separated by SEC (Fig. 3C). SDS-PAGE under non-reducing conditions showed that the material obtained was very pure and consisted mainly of the ≤30 k band and little of the 16 k monomer (Fig. 3D). However, after reduction only the monomeric band was detectable (Fig. 3D). Pancreatic TFF2 purified in this manner was then subject to linear MALDI mass spectrometry as well as LC-ESI-MS/MS analysis.

Characterization of pancreatic TFF2 by LC-ESI-MS/MS analysis and mass spectrometry

The Coomassie stained 15k-band (B15) excised from a reducing gel prepared in parallel to that shown in Fig. 3D (fraction 3B) was subjected to in-gel digestion followed by LC-ESI-MS/MS for protein identification. The major results are summarized in Table 1. Mature porcine TFF2 was identified with a glutamic acid residue at position 61 (61E, corresponding to 86E in the precursor) and an alanine residue at position 80 (105A in the precursor).

FPLC-purified TFF2 (fraction 3B from Fig. 3D) was directly analyzed using linear MALDI mass spectrometry to determine the molecular mass of the intact protein. The mass determined using internal calibration markers was 11713.5 (Fig. 3E).
Porcine pancreatic TFF2 forms a SDS resistant homodimer

As clearly seen after SEC, TFF2 from the porcine pancreas forms a low molecular mass product (Fig. 1B). Surprisingly, pancreatic TFF2 appears on a non-reducing SDS-PAGE mainly by absorbance at 280 nm. The TFF2-positive fractions used for further purification are shown in grey. (C) Elution profile after separation of the TFF2-positive Resource Q6 fractions (see B) on a Superdex 75 resin as determined by absorbance at 280 nm. The TFF2-positive fractions No. 37-39 used for further analysis are shown in grey. (D) 15% SDS-PAGE of fractions 37-39 (see C) under reducing and non-reducing conditions (post-in-gel reduction) and subsequent silver staining. The molecular mass standard is indicated on the left. (E) MALDI mass spectrometry of purified TFF2 (fraction No. 38 after separation on Superdex 75 HL, see C). The sample was mixed with the calibration standard and analyzed as described in the methods section.

Discussion

Porcine pancreatic TFF2 forms a SDS resistant homodimer

As clearly seen after SEC, TFF2 from the porcine pancreas forms a low molecular mass product (Fig. 1B). Surprisingly, pancreatic TFF2 appears on a non-reducing SDS-PAGE mainly...
in the range of ≤30 k with a minor band of about 16 k, the latter representing the TFF2 monomer (Fig. 1D). After reduction, only monomeric TFF2 was observed (Fig. 1C). Thus, the ≤30 k band was expected to represent a TFF2 homodimer, which has not been described in solution thus far. However, porcine TFF2 is well known to crystallize as a non-covalently linked dimer [4, 46, 47]. Generally, a TFF2 dimer is reminiscent to the xP4 peptides from *Xenopus laevis* stomach containing four TFF domains in tandem [43].

Noteworthy, the ≤30 k form of TFF2 was immunologically detectable on a non-reducing SDS-PAGE only after post-in-gel reduction (Fig. 1D). If the post-in-gel reduction step was omitted, the antibody did not detect this form any more (data not shown). This might be due to the disulfide bridge between cysteine-6 and cysteine-104 [4], which could mask the antigenic epitope recognized by the antiserum anti-hTFF2-1.

LC-ESI-MS/MS analysis revealed a new TFF2 variant characterized by 61E and 80A in the mature peptide. The 61E variant is in agreement with the predicted sequence after cDNA cloning [8], but differs from the reported protein sequence (61Q) [3]; whereas 80A is congruent with the reported protein sequence [3], but differs from the sequence predicted from cDNA cloning (80R) [8].

The molecular mass of the ≤30 k band was determined to be 11713.5 (Fig. 3E), which is fully compatible with the 61E/80A variant of a TFF2 monomer after cyclization of the N-terminal glutamine residue and formation of 7 disulfide bridges (calculated molecular mass: 11713.1). This result indicates that the ≤30 k band consists of TFF2 monomers, which non-covalently associate probably to a dimeric form. This is unusual because the non-covalent linkage is partly resistant to boiling SDS during sample preparation for the non-reducing SDS-PAGE (Fig. 1D). However, a similar situation has been described, e.g., for MHC class II molecules [48].

**Gastric TFF2 is non-covalently linked to MUC6**

TFF2 from porcine stomach differs markedly from that of the porcine pancreas because it forms a high molecular mass complex (Fig. 1A) as similarly described for human [15, 24].

When the high molecular mass complex of TFF2 was isolated from antral mucosal scrapings (Fig. 2A) and then separated by anion-exchange chromatography (Fig. 2B), the TFF2 positive material was heterogeneous and broadly split between fractions 17 and 41 (Fig. 2B). This is in sharp contrast to pancreatic TFF2, which appeared as a sharp peak (fractions 18/19; Fig. 3B). Thus, gastric TFF2 seemed to be bound to high molecular mass proteins, e.g., mucins, which apparently occur in a broad range of different isoelectric variants probably due to several glycoforms. A systematic analysis of the FPLC fractions by SDS-AgGE clearly revealed that TFF2 occurred mainly in an ultrahigh molecular mass form, which did not even enter the agarose gel (Figs. 2C, 2D). A nearly congruent pattern was obtained for binding of the lectin GSA-II (Figs. 2C, 2D), which specifically recognizes the mucin MUC6 [44]. Thus, TFF2 seems to be predominantly associated with the mucin MUC6. This is in line with the fact that TFF2 and MUC6 are synthesized by the same cells and co-localize within the laminated gastric mucus layer [9, 10, 20, 21]. In contrast, the mucin MUC5AC appeared mainly in fractions 18-22 with variable molecular mass considerably lower than that of TFF2 and MUC6 (Figs. 2C, 2D). The lower M<sub>r</sub> of MUC5AC when compared with that of MUC6 is in agreement with previous reports [44, 49]. Thus, the two TFF domains in TFF2 would be perfectly designated to crosslink MUC6, which might be the reason for the higher M<sub>r</sub> when compared with MUC5AC.

Under reducing conditions, gastric TFF2 was completely released in monomeric form from the high molecular mass complex (Fig. 1C). In contrast, under non-reducing conditions, two TFF2 forms were observed (Fig. 1D), i.e., monomeric TFF2 as well as a high molecular mass complex, which hardly entered the stacking gel. This indicates that TFF2 is bound to a high molecular mass component (probably MUC6) clearly in a non-covalent fashion and maybe also by a covalent linkage forming a disulfide-linked heteromer. The latter would be reminiscent to that of the TFF1-GKN2 [24, 50] and TFF3-FCGBP heteromers [42]. However, porcine TFF2 contains an even number of cysteine residues forming seven disulfide bridges;
thus, at least one disulfide bridge in TFF2 must be opened in order to enable formation of a heteromer. The most likely candidate would be the linkage between Cys-6 and Cys-104 because this disulfide bridge has been reported to be particular sensitive to reduction with glutathione [51]. As a consequence, TFF2 could then covalently crosslink, e.g. MUC6, either in an intra- or an intermolecular fashion. The latter architecture would establish TFF2 as a covalent link peptide. Alternatively, gastric TFF2 could also interact non-covalently with MUC6 in a manner, which is resistant to boiling SDS similar as the pancreatic TFF2 homodimer. However, both concepts still await further experimental validation.

Generally, the non-covalent and preferential binding of TFF2 to MUC6 could be due to a lectin activity of TFF2 recognizing a specific carbohydrate moiety present in MUC6 but not MUC5AC. For example, the carbohydrate moiety of MUC6, and not MUC5AC, contains the structure GlcNAcα1→4Galβ1→R at its non-reducing end [52], which is specifically detected by the lectin GSA-II [44], the monoclonal antibody HIK1083 [52] and the paradoxical concanavalin A staining [53]. Of note, based on its crystal structure a lectin activity has already been predicted for TFF2 enabling the non-covalent cross-linking of mucins [4]. This hypothesis is strongly supported by our results now.

Cross-linking of mucins – both by covalent and non-covalent interactions - is particularly important for the assembly of the laminated structure and the rheological properties of gastric mucus. The latter has already been proven experimentally [25, 26]. Thus, it is an important goal now to clarify the chemical nature of the non-covalent interaction between TFF2 and MUC6.

Acknowledgements

The authors thank A. Kohnke and E. Voß for their excellent technical and secretarial assistance, respectively, H. Ragge for his support at the initial phases of this project, Prof. J. R. Davies (Malmö) for providing the PGM2B antiserum, and Dr. J. Lindquist for careful proofreading of the manuscript. MS analyses were performed from the Center for Molecular Medicine Cologne.

References


18 Ermund A, Schute A, Johansson ME, Gustafsson JK, Hansson GC: Studies of mucus in mouse stomach, small intestine, and colon. I. Gastrointestinal mucus layers have different properties depending on location as well as over the Peyer’s patches. Am J Physiol 2013;305:G341-347.


26 Kjelley S, Nexo E, Thim L, Poulson SS: Systemically administered trefoil factors are secreted into the gastric lumen and increase the viscosity of gastric contents. Br J Pharmacol 2006;149:92-99.


37 Graness A, Chwieralski CE, Reinhold D, Thim L, Hoffmann W: Protein kinase C and ERK activation are required for TFF-peptide-stimulated bronchial epithelial cell migration and tumor necrosis factor-α-induced interleukin-6 (IL-6) and IL-8 secretion. J Biol Chem 2002;277:18440-18446.


50 Westley BR, Griffin SM, May FEB: Interaction between TFF1, a gastric tumor suppressor trefoil protein, and TFIIZ1, a brichos domain-containing protein with homology to SP-C. Biochemistry 2005;44:7967-7975.

