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Seminal pepsinogen C is not identical with, but is very similar to gastric pepsinogen C

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Human seminal pepsinogen C has been purified and compared with gastric pepsinogen C. The two zymogens cannot be distinguished by amino acid compositions and sequences of the first 28 N-terminal amino acid residues are identical. Apparent immunological identity is observed with polyclonal antisera. Monoclonal antibodies toward seminal pepsinogen C have been produced. One is able to recognize a non-carbohydrate antigenic determinant only present in seminal pepsinogen C.

Pepsinogen C; Amino acid composition; N-terminal sequence; (Human semen)

1. INTRODUCTION

Pepsinogens are mainly synthesized in the stomach, but extragastric zymogens have also been observed. Lundquist and Seedorff [1] demonstrated that human seminal plasma contains a zymogen with properties related to a gastric pepsinogen. This was later shown to be pepsinogen C [2]. Recently, we have found that the α-cells of the pancreatic islets contain pepsinogen C (PgC) [3]. By agar gel electrophoresis at pH 6.0, 3 components of PgC with different electrophoretic mobilities are found. All have apparent immunochemical identity with polyclonal antibodies [4]. The two fastest moving components are observed in semen [2,4]. Apparent immunological identity is also observed among seminal, pancreatic and gastric PgC [2–4]. But the published amino acid compositions of seminal and gastric PgC show differences, especially in the contents of basic amino acid residues [5–7]. This apparent discrepancy prompted us to isolate and analyze the human seminal PgC.

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Abbreviations: PgC, pepsinogen C (EC 3.4.23.3); SDS-PAGE, SDS-polyacrylamide gel electrophoresis; CBB R-250, Coomassie brilliant blue R-250; FPLC, fast-protein liquid chromatography; PTH, phenylthiohydantoin

2. MATERIALS AND METHODS

2.1. *Seminal PgC*

Pooled samples of semen were obtained from the Infertility Clinic, Hvidovre Hospital, and from the Laboratory for General Practitioners (Copenhagen). After centrifugation (20000 × *g*, 5°C for 30 min) of a 200 ml pool of semen, the pellet was washed with 200 ml of 0.05 M sodium phosphate, pH 6.0. Centrifugation was repeated. The combined supernatants were filtered through a Whatman no.1 filter and dialyzed against 0.05 M sodium phosphate, pH 6.0. Purification: step 1, ion-exchange on a 25 × 300 mm column of DE-32 cellulose (Whatman), equilibrated with 0.05 M sodium phosphate, pH 6.0, and eluted with a linear gradient of 400 ml of equilibration buffer plus 400 ml of 0.4 M sodium phosphate, pH 5.6. Step 2, zymogen containing fractions were diluted for absorption on a 10 ml column of DE-32 cellulose, a concentrated solution was obtained by elution with 20 ml of 0.4 M sodium phosphate, pH 5.6, containing 1 M sodium chloride. Third step, gel filtration on a 25 × 900 mm column of Sephadex G-100 (Pharmacia) equilibrated and eluted with 0.05 M ammonium acetate, pH 5.6. Step 4, FPLC ion-exchange on a Mono Q 5/5 column (Pharmacia) equilibrated with 0.05 M

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ammonium acetate, pH 5.6, elution over 30 min (flow rate of 1 ml/min) with a linear gradient of ammonium acetate, pH 5.6, to 0.4 M.

2.2. *Assays*

The purification was monitored by fused rocket immunoelectrophoresis with polyclonal anti-gastric PgC raised in rabbits [8], by SDS-PAGE and by assay of proteolytic activity carried out with radial diffusion in skim milk containing agarose gels with activated gastric PgC as standard [9].

2.3. *Gastric PgC*

PgC was prepared from human gastric mucosa by anion-exchange chromatography [7].

2.4. *Production of monoclonal antibodies*

Female Balb/c mice were immunized by intraperitoneal injections of seminal PgC adsorbed on aluminum hydroxide. Fusion with X63-Ag8.653 myeloma cells was performed with polyethylene glycol 3000 [10]. At 14 days following fusion, the hybridoma supernatants were screened for PgC antibody production by indirect ELISA with seminal and gastric PgC. Positive clones were further investigated for reactivity in Western blot of SDS-PAGE of gastric and seminal PgC.

2.5. *Deglycosylation*

Incubation of 20 μg seminal PgC in 100 μl of 0.05 M sodium acetate, pH 5.0, 1% (v/v) SDS and 5% (v/v) 2-mercaptoethanol was performed overnight at 37°C with 0.25 U endoglycosidase F or with 5 mU endoglycosidase H (Boehringer Mannheim). The reaction mixture was boiled for 5 min in SDS sample buffer and submitted to SDS-PAGE in duplicate. One half was stained with CBB R-250, the other was blotted to nitrocellulose and incubated with the supernatant from hybridoma no.8G7.

2.6. *Amino acid analysis and amino terminal sequence determination*

The amino acid composition of PgC was determined after hydrolysis at 110°C for 24 and 72 h in redistilled 6 M HCl containing 0.1% phenol in evacuated tubes sealed after flushing with nitrogen. The hydrolysate was analyzed on a Waters amino acid analyzer operated with non-halide buffers and post column reaction with *o*-phthaldialdehyde.

Automatic sequencing was carried out using an Applied Biosystems 477A sequencer with an on-line PTH analyzer model 120A.

3. RESULTS AND DISCUSSION

PgC was purified 119 times with an overall recovery of 23% (table 1). The resulting protein showed a single band on SDS-PAGE with an M_r of 40000.

The supernatants from 345 hybridomas were screened by ELISA, 10 clones produced antibodies against seminal PgC, of which 6 also reacted with gastric PgC. One clone (no.8G7) produced an antibody, that bound to seminal PgC in Western blot after SDS-PAGE, but not to gastric PgC.

Table 1

Purification scheme of PgC from seminal fluid

Step	Total protein (mg)	Total pepsinogen C activity (mg)	Degree of purification (μg PgC/ml per A_{280})	Yield (%)
1. Crude	4646	35.7	7.69	100
2. DE-32 (ion-exchange)	153	25.2	165	70.5
3. DE-32 (conc.)	129	18.1	141	50.7
4. Sephadex G-100	18.8	9.6	509	26.8
5. Mono Q	9.0	8.2	915	23.1

Polyclonal antibodies against gastric PgC recognize determinants common to both gastric as well as seminal PgC, as illustrated in fig.1A. The monoclonal antibody (no.8G7), was able to bind to a non-occupied determinant present on seminal PgC only (fig.1B). Pancreatic PgC behaves the same way as the seminal when antibody no.8G7 is employed. Another monoclonal antibody no.7C8 was able to detect faint spurs of non-occupied determinants in the immunoprecipitates produced by the polyclonal antiserum (fig.1C). This means that seminal and pancreatic PgC both have at least one common antigenic determinant which is different from the gastric zymogen.

Digestion of seminal PgC with endoglucosidase H or F did neither influence the mobility in SDS-PAGE nor the binding of antibody no.8G7. This indicates that the epitope is not a carbohydrate moiety.

Within the experimental error, no differences in composition were revealed by amino acid analyses of seminal and gastric PgC carried out in parallel (table 2). This amino acid composition is in agreement with the deduced composition from both cDNA [12] and from genomic DNA [13], and do not support the results previously reported [5,6].

Determination of the N-terminal amino acid sequence of seminal PgC gave the following result: Ala-Val-Val-Lys-Val-Pro-Leu-Lys-Lys-Phe-Lys-Ser-Ile-Arg-Glu-Thr-Met-Lys-Glu-Lys-Gly-Leu-Leu-Gly-Glu-Phe-Leu-Arg. The sequences differ at positions 24 and 25 from that reported previously after amino acid sequencing of gastric PgC [7].

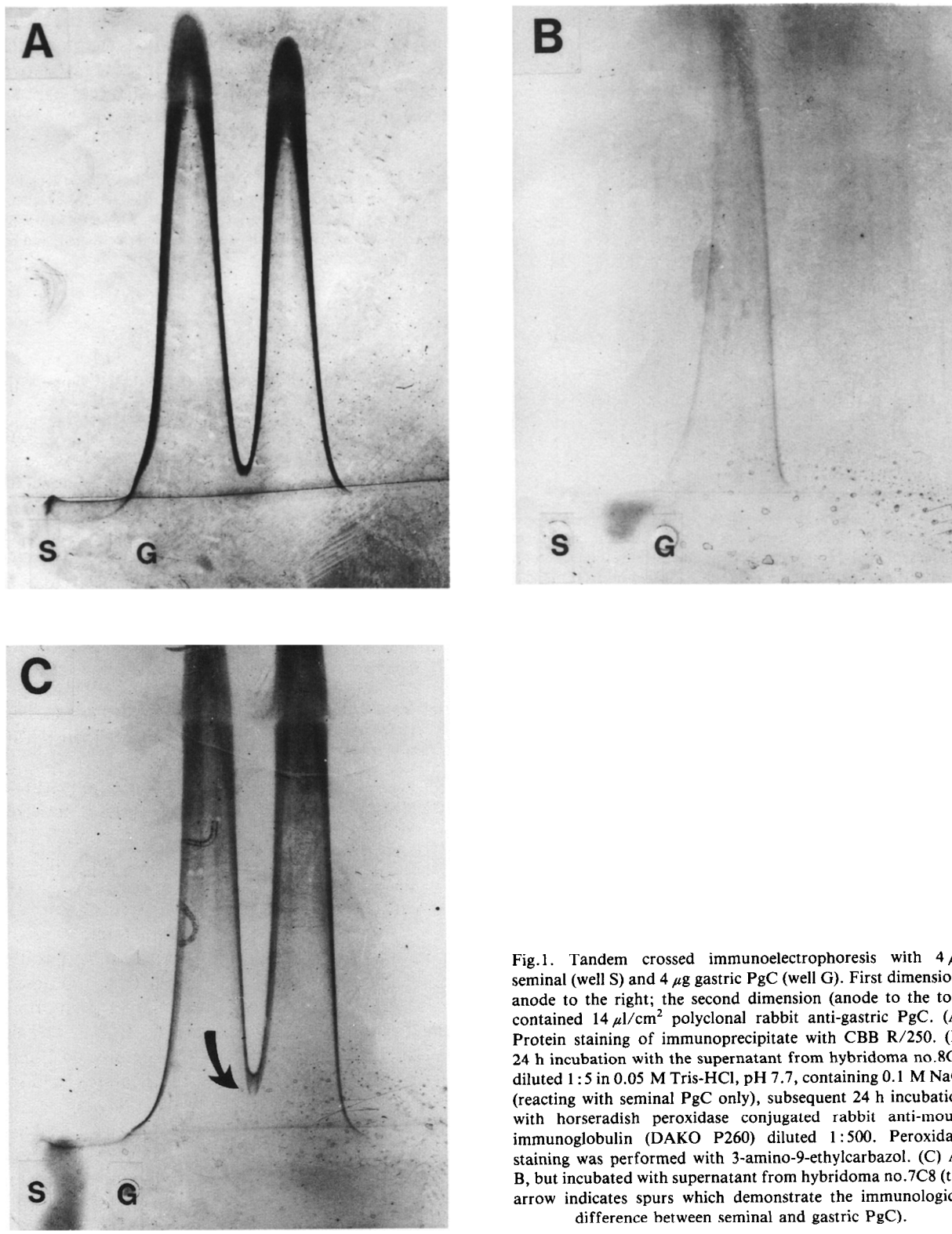

Fig.1. Tandem crossed immunoelectrophoresis with 4 μg seminal (well S) and 4 μg gastric PgC (well G). First dimension, anode to the right; the second dimension (anode to the top) contained 14 μl/cm^2 polyclonal rabbit anti-gastric PgC. (A) Protein staining of immunoprecipitate with CBB R/250. (B) 24 h incubation with the supernatant from hybridoma no.8G7 diluted 1:5 in 0.05 M Tris-HCl, pH 7.7, containing 0.1 M NaCl (reacting with seminal PgC only), subsequent 24 h incubation with horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin (DAKO P260) diluted 1:500. Peroxidase staining was performed with 3-amino-9-ethylcarbazol. (C) As B, but incubated with supernatant from hybridoma no.7C8 (the arrow indicates spurs which demonstrate the immunological difference between seminal and gastric PgC).

Table 2

Amino acid compositions of seminal and gastric PgC

Amino acid	Residues per mol (M_r 40552)		
	a	b	c
Asx	29.8	29.9	29
Thr	24.7	26.2	26
Ser	37.6	37.8	39
Glx	46.0	46.4	45
Pro	19.1	19.2	19
Gly	37.4	38.8	38
Ala	22.3	23.0	22
Val	26.8	26.5	27
Met	6.6	6.5	7
Cys	6.9	5.9	6
Ile	14.7	14.4	15
Leu	33.5	33.3	33
Tyr	21.6	20.6	23
Phe	20.1	19.9	21
His	2.2	2.1	2
Lys	9.2	8.9	8
Arg	6.4	6.7	6
Trp	ND	ND	6

(a) Seminal pepsinogen C (present study); (b) gastric pepsinogen C (present study); (c) deduced from nucleotide sequences from cDNA [12] and genomic DNA [13]. ND, not determined

We therefore repeated the amino acid sequencing with a new preparation of gastric PgC and found the same sequence as that of seminal PgC and the DNA-derived sequences [12,13].

The comparison between gastric and seminal PgC shows that the similarities are predominant. The two zymogens cannot be distinguished by N-terminal amino acid sequence or by amino acid composition. Polyclonal antisera show apparent immunological identity, but with monoclonal antibodies a clearcut difference is observed. This difference is also present after treatment with endoglycosidase H and F. This opens the possibility that the two zymogens are products of two closely related genes which have different expressions in the tissues.

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