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High-performance liquid chromatography: purification and chromatographic behaviour of molecular variants of pepsinogen A from human urine

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ABSTRACT

By combining conventional DEAE chromatography with high-performance liquid chromatography on Sephacryl S-200 HR and Mono-Q columns, we have been able to isolate and fractionate human pepsinogen A (PGA) isozymogens from large amounts of urine. This method of fractionation is simple and allows one to obtain pepsinogen in a native non-denatured conformation. The isozymogens are homogeneous by electrophoretic and chromatographic criteria; this was confirmed by N-terminal amino acid sequencing. Purified PGA-3 and PGA-5 can be converted into an additional, more anionic, isoform on incubation at 37°C. This isoform exists not only *in vitro* but also *in vivo*. The net negative charge of the PGA isozymogens is in the order PGA-5 < deamidated PGA-5 < PGA-3 < deamidated PGA-3. Surprisingly, the elution order on the Mono-Q column was PGA-5/PGA-3/deamidated PGA-5/deamidated PGA-3. We have performed molecular modelling on PGA to investigate this phenomenon in terms of surface charge (not net charge) of the proteins. The model provides evidence that (1) only a fraction of the protein surface interacts with the support and (2) regions of localized charge at the protein surface may allow portions of the external surface to dominate chromatographic behaviour, resulting in a steering of the proteins with respect to the oppositely charged matrix. Pepsinogens may serve as model proteins for elucidating some of the variables that determine the chromatographic behaviour of proteins on ion-exchange columns.

INTRODUCTION

The human gastric proteinases of the pepsinogen A (PGA) multigene family are synthesized as zymogens. Following exocytosis, gastric acid catalyses a conversion reaction resulting in the formation of active enzymes [1,2]. After non-denaturing polyacrylamide gel electrophoresis (PAGE), PGA reveals a multi-banded pattern consisting of two to five isozymogens, designated 2–5 and 5S in decreasing order of anodal mobility [3–5]. With the help of monoclonal antibodies, four different primary gene products (allozymogens) have been identified [4]. Immunochemically they can be divided into α-PGA (PGA-3 and PGA-5) and β-PGA (PGA-4 and PGA-5S) [4]. One of the allozymogens (PGA-5S) is only rarely seen, and sequence data are presently not available. PGA-2 is the second-

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ary product of PGA-3 [4,5] and thus is not an allozymogen but an isozymogen.

In humans, the PGA isozymogens are of potential interest, not only from a diagnostic point of view [6–9] but also because of their molecular evolution [10,11] and differential expression [12]. PGA isozymogens consist of a single polypeptide chain of 373 amino acids, with a calculated molecular mass of 40 300 [13] and an isoelectric point between 4.5 and 4.8 [14]. The PGA multigene family is of a young evolutionary age, that is, all PGA variants are derived recently from a common ancestral gene [10,11]. Consequently, the variants resemble each other very much, as only a few mutations could occur in the primary structure after the duplication of the genes. Variants of PGA differ by only 0.1 pH unit in their isoelectric point [14] and only five amino acid substitutions are mapped in the main PGA variants PGA-3 -4 and -5 [11] (see also Table I). Apart from genetically controlled variants, posttranslational modifications (most probably deamidation) have been reported [4,5], expanding the heterogeneity of the proteins in question. Human pepsinogens are not phosphorylated, and most probably none of the pepsinogens is glycosylated [2].

Several chromatographic separations using homogenates from human gastric mucosal have been reported (*e.g.* refs. 2, 4, 5 and 15). However, now that the majority of ulcer operations are vagotomies, it is difficult to obtain enough stomach mucosa for the separation procedure. Since urine contains a relatively large amount of PGA (200–1000 μg/l), owing to high glomerular sieving and low tubular reabsorption [14], it is worthwhile to develop a separation method for this easily obtained source. As far as we know, from 1980 onwards only two attempts have been described, using conventional methods of protein purification [16,17]. A major drawback of both published methods is that they are either lengthy, requiring several days for elution of the proteins, or very expensive because of the use of ampholytes as spacers for preparative isotachophoresis. Furthermore, only part of the PGA isozymogens could be separated from each other.

TABLE I

DIFFERENCES OF THE PGA ALLOZYMOGENS ACCORDING TO EVERS *et al.* [11] AND THEIR DEAMIDATED PRODUCTS

Isozymogen	Residue number					
	43	77	207	250	310	338
PGA-3	Glu	Val	Gln	Ala	Asn	Leu
Deamidated PGA-3	Glu	Val	Gln	Ala	Asp	Leu
PGA-4	Glu	Leu	Lys	Thr	Asn	Val
Deamidated PGA-4	Glu	Leu	Lys	Thr	Asp	Val
PGA-5	Lys	Leu	Gln	Ala	Asn	Leu
Deamidated PGA-5	Lys	Leu	Gln	Ala	Asp	Leu

This paper describes a three-step procedure using conventional DEAE chromatography combined with high-performance liquid chromatography (HPLC) on Sephacryl S-200 HR and Mono-Q columns to obtain highly purified fractions of the PGA isozymogens. Interestingly, the elution order of the isozymogens on the Mono-Q column did not follow the order of their net negative charge. We have performed molecular modelling on PGA to investigate this phenomenon in terms of the surface charge (not the net charge) of the proteins.

EXPERIMENTAL

Chromatography of urinary pepsinogens

Urine was obtained from a single subject (phenotype BD > BB) suffering Menetrier illness (enteropathia exsudativa of the stomach). Urine (5650 ml, stored for one week at 4°C with 0.02% sodium azide, PGA concentration 6 mg/l) was collected during five consecutive days, and dialysed overnight against 30 l of tap water at 4°C. Next 125 ml of DEAE-Sephacel (Pharmacia, Uppsala, Sweden), washed with 0.025 *M* Bis-Tris–HCl pH 7.0 (solvent A), was added to the dialysed urine and left overnight at 4°C with non-magnetic stirring. After sedimentation for 4 h the supernatant was discarded; the muddy mixture obtained was packed in a column (16.0 × 2.6 cm I.D.) and washed once with solvent A. Proteins were eluted at a flow-rate of 3.8 ml/min in two steps: first, with solvent A containing 0.1 *M* NaCl, and second, after the baseline had returned to its original position, with solvent A containing 0.6 *M* NaCl. Pepsinogen-containing fractions, determined with a milk-clotting assay according to ref. 18, were present in the second elution peak and were pooled (45 ml). After filtration through a FP 030/3 filter (Schleicher & Schuell, Dassel, Germany) fractions of 7.5 ml were applied to an XK26 column packed with Sephacryl S-200 HR (58.0 × 2.6 cm I.D.) (Pharmacia) operated on an FPLC apparatus with a P-500 pump. Proteins were eluted from the column with solvent A at a flow-rate of 3.5 ml/min. Each run expanded the volume of the pepsinogen-containing pool to 32 ml. From this pool, 7-ml fractions were loaded on a Mono-Q HR5/5 column (Pharmacia). An FPLC apparatus consisting of two P-500 pumps and a GP-250 gradient programmer was used to prepare the gradients. After washing with 1 ml of solvent A and 3 ml of 20% solvent B (solvent A + 1 *M* NaCl), pepsinogens were eluted from the column with a linear gradient of solvent B (20 to 35%) at a flow-rate of 1.0 ml/min over 16 ml. The column was cleaned with 4 ml of 100 % solvent B and equilibrated with 5 ml of solvent A. After rechromatography of the separate PGA-containing peaks, the pools were tested for their isozymogen content with PAGE (see below).

Criteria of purity

The homogeneity of the rechromatographed pepsinogens was checked by size-exclusion chromatography and amino acid sequencing. Chromatography on a

Superose 12 HR10/30 column (Pharmacia) was performed on the FPLC system described above; only one pump was used. The column was equilibrated with 0.1 *M* NH_4HCO_3 (pH 7.8); 0.5 ml was applied to and eluted from the column at a flow-rate of 0.8 ml/min. The pepsinogen content was measured with a Shimadzu UV-160 spectrophotometer at 280 nm using an extinction coefficient of 12.5 [19]. N-Terminal amino acid sequencing of 40–60 μg of protein was carried out with the manual liquid-phase dimethylaminobenzene isothiocyanate–phenyl isothiocyanate (DABITC–PITC) double-couple procedure, as described by Chang [20].

Non-denaturing PAGE

PAGE was performed essentially according to Taggart and Samloff [4] and Frants *et al.* [3]. Vertical polyacrylamide slab gels (16 × 20 × 0.15 cm) were soaked with 75 ml of acrylamide–N,N'-methylenebisacrylamide (T = 8.3%, C = 3.0%) in 35 m*M* Tris–HCl (pH 7.5). After deaeration, polymerization was performed for at least 45 min after addition of 50 μl of TEMED and 250 μl of 10% (w/v) ammonium peroxodisulphate. Stacking gels consisted of 22 ml of acrylamide–N,N-methylenebisacrylamide (T = 3.6%, C = 20.0%) in 30 m*M* Tris–H_3PO_4 (pH 5.0) with 0.67 *M* sucrose. Photopolymerization was catalysed by 20 μl of TEMED and 3 ml of riboflavin (4 mg per 100 ml) for 1.5 h. The upper and lower reservoir were filled with 2.2 m*M* Tris–15 m*M* diethylbarbituric acid (pH 7.0). Samples were diluted 2:1 with 40% sucrose in 30 m*M* Tris–H_3PO_4 (pH 5.5) prior to application. Electrophoresis was performed at a constant voltage of 300 V until the tracking dye, Bromophenol Blue, entered the separating gel (*ca.* 30 min) and continued for 2 h at a constant voltage of 900 V. Electrophoresis was stopped after the tracking dye entered the lower reservoir. Staining for proteolytic activity was performed by soaking the gel for 15 min in a solution of 1% bovine haemoglobin in 0.1 *M* HCl at 37°C, followed by a 40-min incubation in 0.1 *M* HCl at 37°C. After incubation, the gels were stained for protein in a solution containing 0.2% Coomassie Brilliant Blue R250 in 10% (v/v) acetic acid–25% (v/v) 2-propanol. After destaining in 10% acetic acid, PGA patterns appeared as white bands on a blue background.

Incubation with monoclonal antibodies

The α-PGA specific antibody 219-20, described by Taggart and Samloff [4], was used in the present study and was a kind gift of Dr. R. T. Taggart (Wayne State University School of Medicine, Detroit, MI, USA). Ascites fluid, containing 11 mg/ml antibody, was diluted ter-fold in phosphate-buffered saline. Samples (50 μl) were incubated for 30 min at 37°C with 5 μl of the antibody dilution prior to electrophoresis. The antigen–antibody complexes were visible in the gel as slow-moving bands.

Molecular modelling

The tertiary structure of human PGA was determined with a comparative

molecular model building method as described previously [21], using the experimentally determined structure of porcine pepsinogen A [22] as the template. In short, the amino acid side-chains of porcine PGA were replaced for the topologically equivalent side-chains of human PGA with the computer program MUTATE (Randy J. Read, University of Alberta). Unacceptable close Van der Waals contacts resulting from this initial building step were detected by the program INTRA (Anita R. Sielecki, University of Alberta). To correct for these contacts, manual adjustments to side-chain torsional angles were made using the program MMS (Steve Dempsey, University of California at San Diego) on a Silicon Graphics IRIS 3030. A two-residue insertion (Pro45-Thr46) was made in the modelled structure with the program FRODO [23] on a Silicon Graphics IRIS 4D70GT, followed by adjustments to the main- and side-chain torsional angles to obtain a stereochemically allowed model. To ensure that all unfavourable contacts were relieved, and to idealize peptide bond geometry in the region of the insertion, steepest descents energy minimization was performed using the CHARMM package [24]. Porcine PGA was minimized in parallel to the modelled structure as control in order to provide a standard by which to judge the results.

RESULTS

Isolation procedure

Because of the large volume of urine needed to obtain sufficient amounts of pepsinogens we chose as a first step a batchwise adsorption of pepsinogen to DEAE-Sephacel. After incubation overnight at 4°C we were not able to detect any pepsinogen in the urine, neither with electrophoresis nor with a sensitive double-sandwich enzyme-linked immunoassay technique (ELISA) [25] (data not shown), indicating that most if not all pepsinogens were adsorbed to the matrix. After elution, the original volume of 5650 ml was reduced to 45 ml. The many proteins present in urine were separated on the basis of their hydrodynamic volume with a Sephacryl S-200 HR column (Fig. 1), then the final purification step was ion-exchange chromatography on a Mono-Q column (Fig. 2). The four isozymogens, present in the urine before the purification procedure, could be separated to homogeneity after rechromatography on a Mono-Q column (same conditions as the preceding run). Following non-denaturing PAGE, the isolated isozymogens showed the same mobility as in urine. Each isozymogen showed on a Superose 12 column a single, sharp, symmetrical peak (Fig. 3). The purity of each sample was checked with N-terminal amino acid sequencing. Three degradation steps were carried out: the expected sequence Ile-Met-Tyr [11] was found in all four cases. Contaminating amino acids were present in the PGA-5 fraction only if the sheets were heavily overloaded; after the second step they disappeared. All isozymogens reacted with monoclonal antibody 219-20.

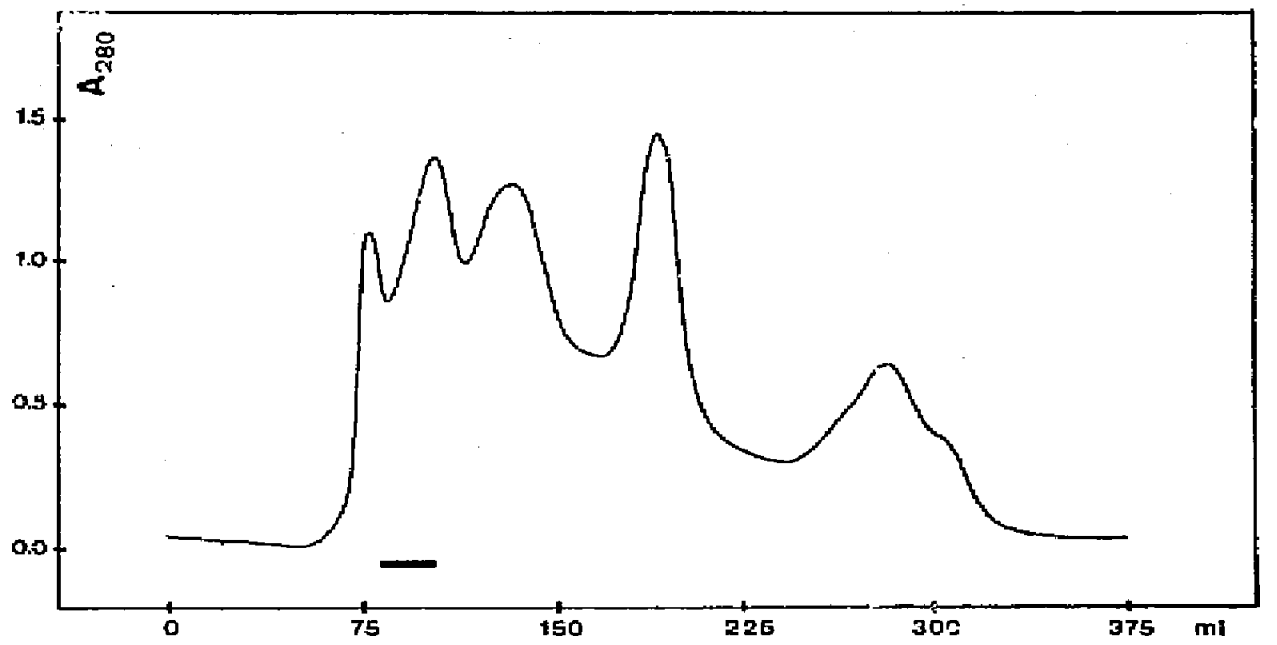

Fig. 1. Sephacryl S-200 HR column chromatography of the pepsinogen concentrate from the DEAE column. A volume of 7.5 ml was applied to 58.0 × 2.6 cm I.D. column; proteins were eluted with 0.025 *M* Bis-Tris–HCl (pH 7.0) at a flow-rate of 3.5 ml/min. The bar indicates the pepsinogen-containing fraction that was further analysed on a Mono-Q column (see Fig. 2).

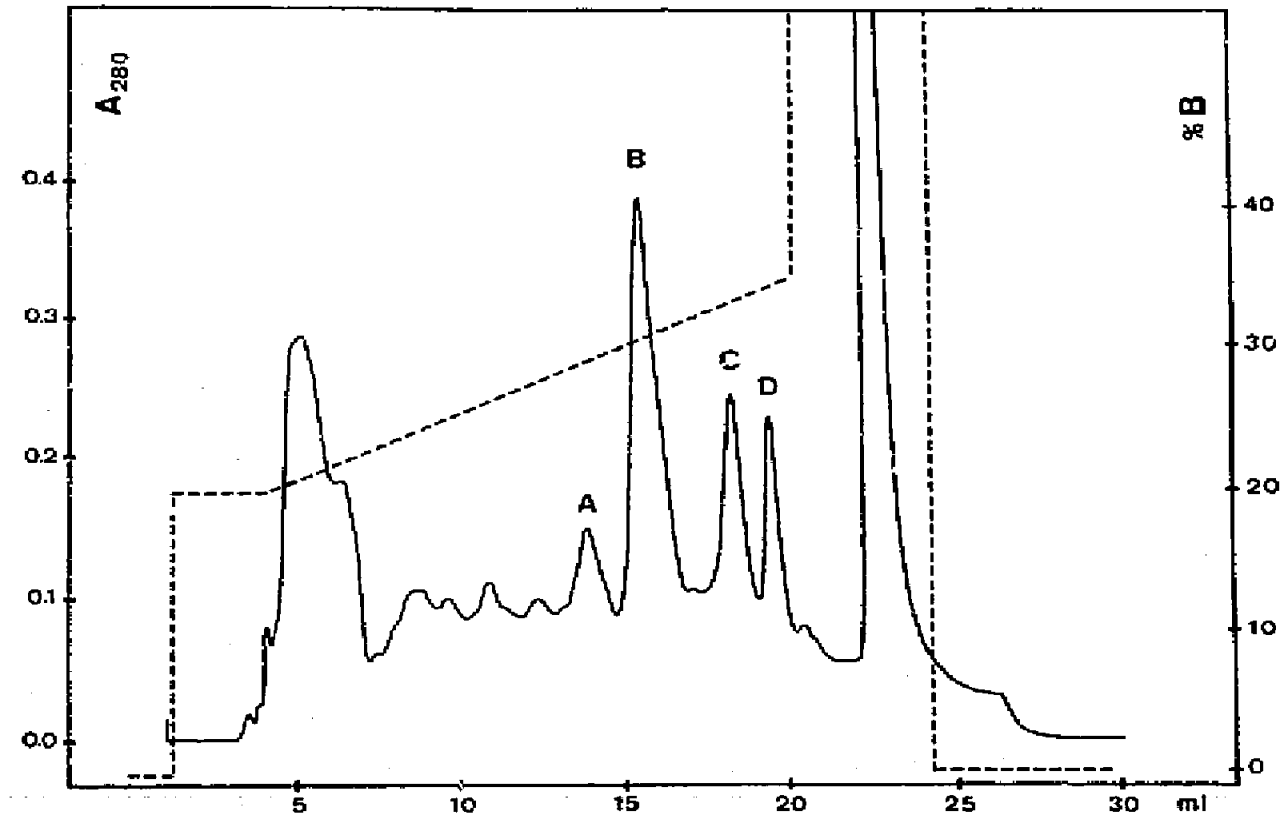

Fig. 2. Purification of pepsinogen A isozymogens by anion-exchange chromatography on a Mono-Q HR5/5 column. Elution was performed with a 16 ml linear NaCl gradient from 0.20 to 0.35 *M* in 0.025 *M* Bis-Tris–HCl (pH 7.0) buffer at a flow-rate of 1.0 ml/min. Peaks: A = allozymogen PGA-5; B = allozymogen PGA-3; C = deamidated PGA-5; D = deamidated PGA-3 (PGA-2).

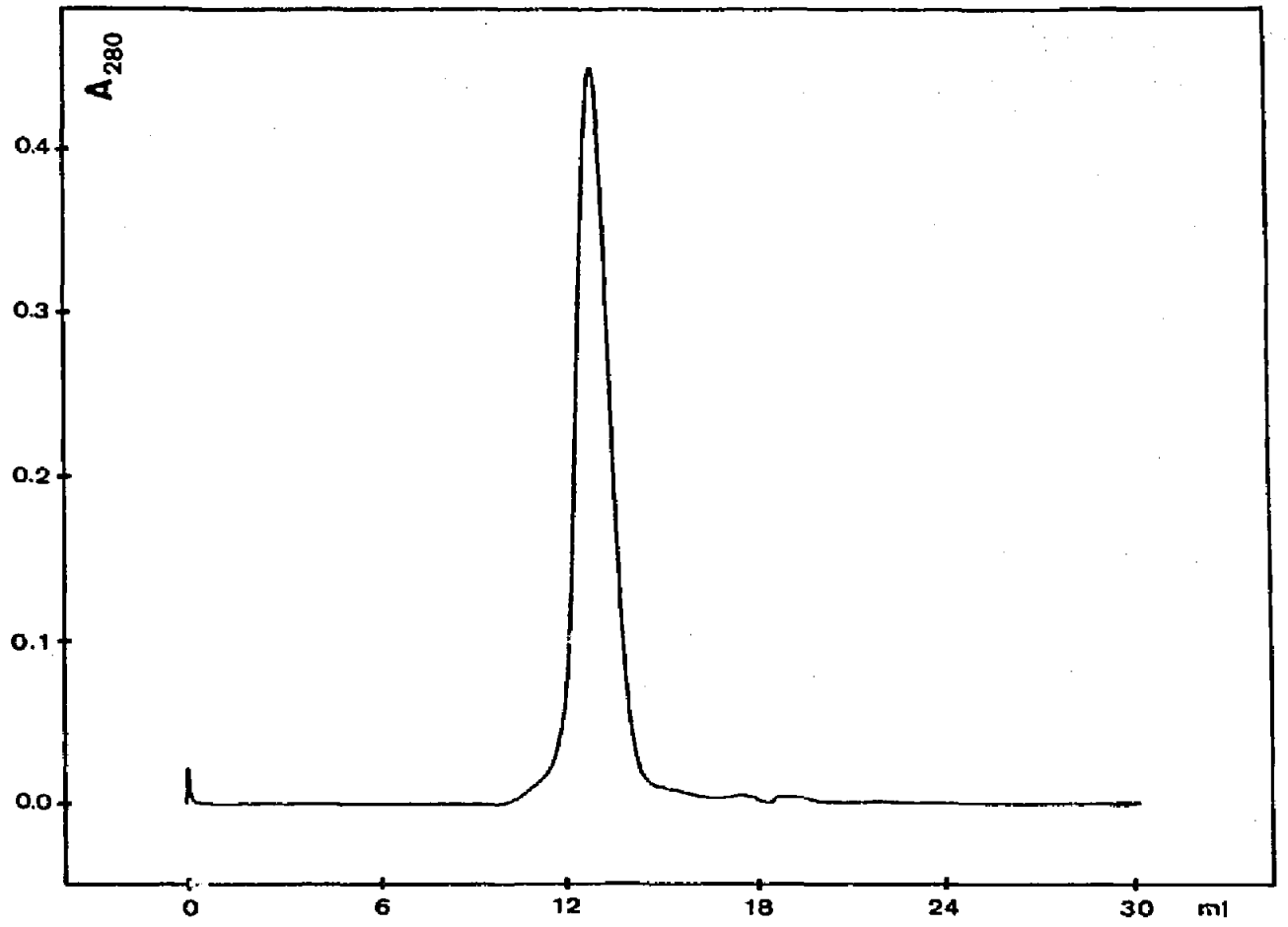

Fig. 3. Purity check of allozymogen PGA-3 with size-exclusion chromatography on a Superose 12 HR 10/30 column. The column was equilibrated in 0.1 *M* NH_4HCO_3 (pH 7.8); the flow-rate was 0.8 ml/min.

Molecular modelling

The polypeptide chain fold (α-carbon atoms only) of porcine and human PGA is given in Fig. 4A. The overall folding and the topology of structural elements of human PGA are presented in Fig. 4B. The localization of the amino acid substitutions between the three major human PGA allozymogens (Table I) are marked with an asterisk (Fig. 4B). The amino acids 77 and 250 are arranged in β-sheets, residue 207 is located in a hairpin bend, and the remaining two residues 43 and 338 are situated in stretches that seem to have few secondary structural features.

DISCUSSION

Phenotyping of PGA patterns

Human PGA is encoded by a multigene family: the variation in PGA phenotypes is due to the occurrence of a number of different PGA haplotypes, each consisting of a different combination and/or number of copies of PGA genes

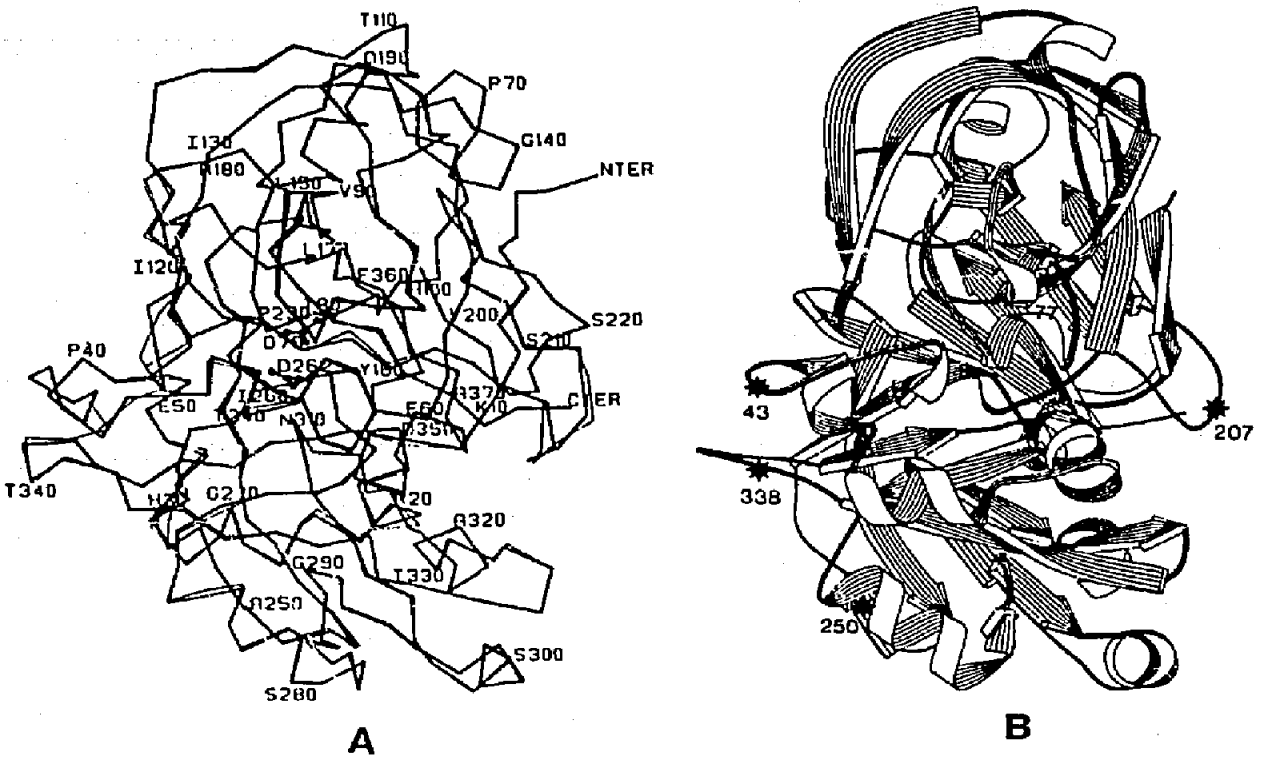

Fig. 4. Two different representations of pepsinogen after energy minimization. (A) Cα carbons and their connecting bonds of human pepsinogen A (thick lines) superimposed on those of porcine pepsinogen A (thin lines). Every tenth residue of human PGA is numbered (sequence number according to ref. 11) and labeled with the residue type (one letter amino acid code). The side-chains of the two aspartic acid residues of the active site are also shown and labeled. NTER = N-terminus; CTER = C-terminus. (B) A Richardson-type drawing showing the overall folding and the topology of structural elements of human pepsinogen A. The view is the same as that in A. The locations of the amino acid substitutions of the allozymogens PGA-3, -4 and -5 are shown with asterisks.

[26–28]. More than twenty phenotypes can be recognized when taking into account not only the presence or absence but also constant differences in relative intensities of the electrophoretic fractions [3]. According to the nomenclature of Frants *et al.* [3], the urinary phenotype of the subject under study is designated AB > BD, as the intensities of the PGA isozymogens are in the order PGA-3 > PGA-5 > PGA-4 (Fig. 5). However, incubation with monoclonal antibody 219-20, recognizing the allozymogens PGA-3 and PGA-5 as well as their more anionic products [4], revealed that all the isozymogens belong to the immunological subclass α-PGA. Thus, the PGA-4 in our subject is not the PGA-4 allozymogen but is in fact the secondary product of PGA-5. This secondary product of PGA-5 co-migrates on electrophoresis with PGA-4 [4,5] (Fig. 5) but elutes, in contrast to PGA-4, from a Mono-Q column behind PGA-3 [5] (Fig. 2). The phenotype of the subject under consideration should therefore be classified as BD > BB. This illustrates clearly that the typing of PGA patterns is more complex than generally thought, and that incubation with 219-20 is in most cases a necessity to determine

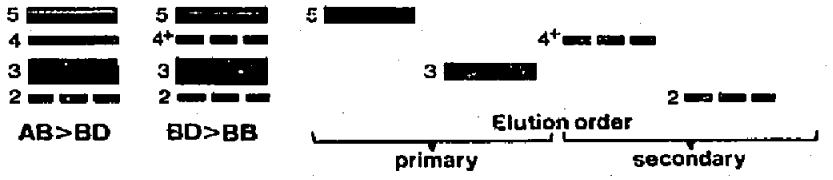

Fig. 5. Schematic representation of the primary (genetic) and secondary (posttranslational) PGA fractions as seen on non-denaturing polyacrylamide gels. On the left, the phenotype of the subject under consideration as determined before (AB > BD) and after (BD > BB) incubation with monoclonal 219.20. On the right, the elution order from the Mono-Q column. The heavy lines represent the primary gene products, the broken lines the posttranslational modifications. 4^+ = deamidation product of PGA-5.

the true phenotype. If this monoclonal antibody is not available, the absence of the primary gene product PGA-4 can also be demonstrated with the Mono-Q column, since the secondary product of PGA-5 elutes after PGA-3 whereas PGA-4 elutes in front of PGA-3 (ref. 5 and this study).

Deamidation of PGA

Conversion of pepsinogens into a more anionic product is observed not only *in vivo* (*e.g.* in the stomach), but also during storage of pure preparations of the allozymogens [4] (see also Fig. 6). Consequently, the conversion is of a non-enzymic nature, leaving spontaneous deamidation as the most probable candidate. It is tempting to speculate which residue is responsible for the conversion of

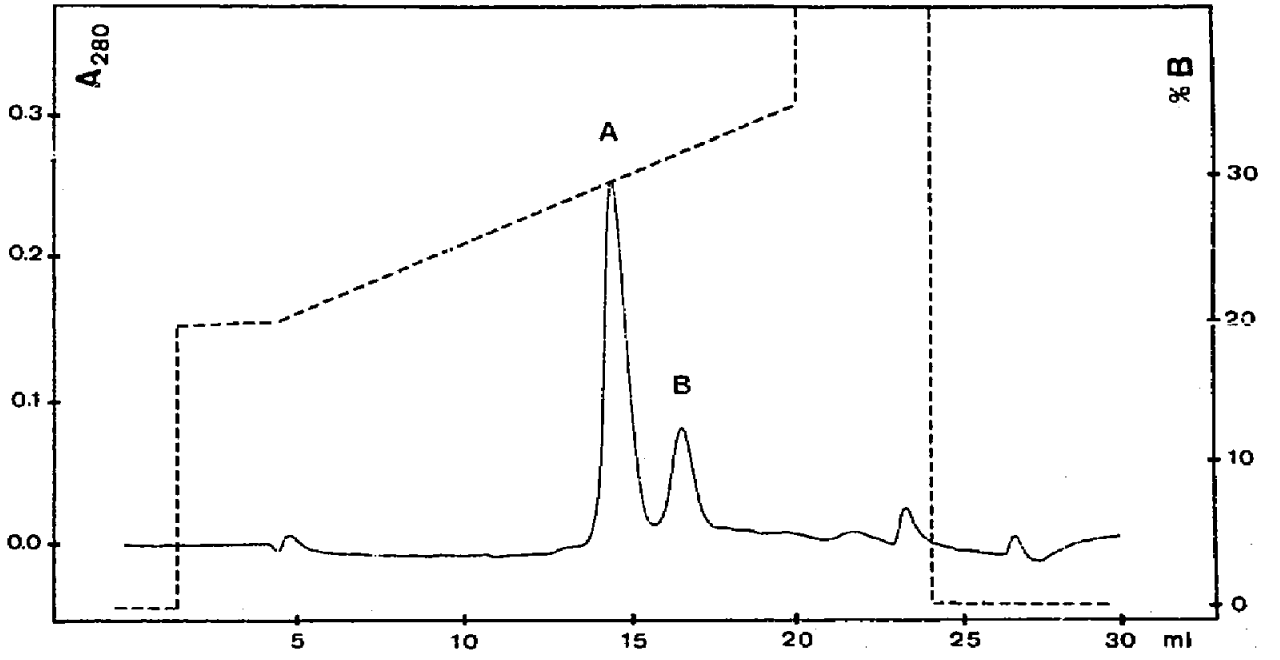

Fig. 6. Deamidation of PGA-3 as revealed by anion-exchange chromatography. Purified PGA-3 was incubated at 37°C for 72 h in 0.025 *M* Bis-Tris–HCl (pH 7.0) containing 0.26 *M* NaCl. The resulting mixture was applied to a Mono-Q column and eluted as described under Fig. 2. Peaks: A = allozymogen PGA-3; B = deamidated PGA-3 (PGA-2). Similar results were obtained for PGA-5 (data not shown).

pepsinogens into a more anionic product. Only glutamine (Gln) and asparagine (Asn) residues undergo deamidation under physiological conditions. Spontaneous deamidation of Asn residues linked to the α-amino group of glycine is well documented for other proteins [29]. Both human PGA and pepsinogen C (PGC), another gastric proteinase, form anionic products under the same conditions (own observations). The only topologically equivalent Asn-Gly combination found in both PGA and PGC is Asn310 (for the residue numbering of PGA and a sequence alignment of PGA and PGC see ref. 30). According to the molecular model this residue is arranged in a β-sheet structure and located on the surface of the molecule. Such an environment makes the residue more susceptible to deamidation [31]. Interestingly, Asn310 is located in a highly conserved stretch; the topologically equivalent Asn of porcine pepsinogen A was found to be deamidated [32]. Thus it is conceivable, but remains to be proved, that Asn310 is responsible for the conversion of human PGA and PGC into a more anionic product.

In contrast to this study, Defize *et al.* [5] reported that the secondary products formed *in vitro* co-eluted with the original isozymogen. However, their incubations were performed at pH 6.0 at 4 and 20°C, whereas the incubation in our study resembled more closely physiological conditions (pH 7.0; 37°C). This may explain their contradictory observations.

Purification of molecular variants of PGA

Although PGA in urine reaches concentrations far exceeding those in serum [14], its amount is generally small. Thus, large volumes of urine are required for enzyme isolation. As urine is easy to obtain and as our procedure is convenient to perform this problem is largely overcome. After the final chromatographic step, the molecular variants PGA-3 and PGA-5 and the deamidation products of PGA-3 and PGA-5 were purified to homogeneity. Non-denaturing PAGE revealed that the isozymogens were still active after purification and that the electrophoretic mobility was unchanged, indicating that no significant alterations in their overall structures had taken place. The same can be concluded from incubations with monoclonal antibody 219-20: all fractions retained their antigenicity after purification. Thus, our procedure enables the separation of PGA isozymogens from urine in a rapid and relatively convenient way without loss of its biological activity.

Chromatographic behaviour of pepsinogens

The remarkable resolving power of the Mono-Q column is evident from the separation of the closely related PGA isozymogens. PGA-3 and PGA-5 differ by only two amino acids (see Table I). The molecular model of PGA revealed that substitution Val77 > Leu77 is buried inside the molecule, whereas substitution Glu43 > Lys43 is located on the surface (Fig. 4B). Thus, the Mono-Q column is able to distinghuish between PGA-3 and PGA-5, proteins of 373 amino acids in length, on the basis of a single amino acid substitution. The same is seen for

PGA-3 and PGA-5 and their deamidated counterpart: the only amino acid that is different is the putative Asn310 > Asp310 conversion.

The net negative charge of the PGA isozymogens is in the order PGA-5 < deamidated PGA-5 < PGA-3 < deamidated PGA-3. If the anion-exchange process is solely based on the sum of electrostatic interactions, *i.e.* the net charge, one should expect the same elution order. However, the following elution order was found: PGA-5/PGA-3/deamidated PGA-5/deamidated PGA-3. Kopaciewicz and co-workers [33,34] have reported a retention model for the anion-exchange process. In brief, the hypothetical model suggested that, under the same chromatographic conditions, (1) for steric reasons only a faction of the protein surface interacts with the support, (2) protein charge asymmetry may cause molecular orientation during adsorption and (3) retention appeared to be direclty proportional to the number of the interacting negatively charged amino acids.

Let us consider PGA-3 and the deamidated product of PGA-5. The former elutes behind PGA-3, and both molecules show the same number of negatively charged amino acids (Table I). However, one negatively charged amino acid differs in respect to its location: Glu43 of PGA-3 is located in the highly positively charged propart of pepsinogen [11], whereas the deamidated residue of PGA-5 shares, with Asp242, Glu255 and Glu249, a region that is free of positively charged amino acids (for a Van der Waals picture of this region see Bank *et al.* [21]). Both are located on the surface of the molecule (Fig. 4B). The retention of both isozymes provides evidence that heterogeneity in the amino acid distribution at the protein surface may allow portions of the external surface to dominate chromatographic behaviour. As intramolecular charge asymmetry promotes differences in electrical potential on the surface of the protein [35], the region of localized charge in which residue 310 is situated orients or "steers" the pepsinogen molecule with respect to the oppositely charged ion-exchange matrix. The chromatographic behaviour of the two molecules therefore supports the first two propositions of the model of Kopaciewicz and co-workers [33,34]. Recent studies on lysozyme and myoglobin also revealed that only a portion of the negatively charged amino acid side-chain groups are involved in the interactive process with the matrix, and that protein-surface interactions in anion-exchange chromatography will be mediated through the negatively charged regions of highest electrostatic potential [36,37].

Evidence for the third proposition is provided by the elution order of PGA-3 and PGA-5. The only amino acid that is located on the surface and is different between PGA-3 and PGA-5 is residue 43. It is a Glu-to-Lys substitution and introduces an extra acidic residue in favour of PGA-3. This residue is therefore responsible for the longer retention time of PGA-3 compared with PGA-5. Similar explanations can be given for the elution order of PGA-3 *versus* deamidated PGA-3 and that of PGA-5 *versus* deamidated PGA-5.

The systematic study of variables that control the chromatographic behaviour of proteins is still in the early stages [38]. This is due to the fact that proteins are

three-dimensional matrices with a great diversity in the distribution of surface functional groups. Significant insight into the structural factors of the ionotopic microstructure (coulombic binding site) of proteins that control or influence the selectivity of chromatographic systems is emerging [36,37,39] but is still not fully elucidated. As PGA isozymogens are highly similar proteins that are well characterized in terms of their biochemistry, pepsinogens may serve as model proteins for elucidating at least some of the variables that determine the chromatographic behaviour of proteins on ion-exchange columns.

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