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Improved detection of human pancreatic proteins separated by two-dimensional isoelectric focusing/sodium dodecyl sulphate gel electrophoresis using a combined staining procedure[a]

MARION HÄNSLER*, G. APPELT and R. ROGOS

Forschungsabteilung Gastroenterologie, Medizinisch-Poliklinisches Institut, Universität Leipzig, Härtelstrasse 16–18, Leipzig 7010 (G.D.R.)

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ABSTRACT

In order to assess secretory pancreatic proteins in a two-dimensional isoelectric focusing/sodium dodecyl sulphate electrophoresis gel, a highly sensitive double-staining method with Coomassie Brilliant Blue followed by silver stain was used. This combined procedure afforded more distinct spots and additional bands, particularly glycoproteins, than either silver or Coomassie Blue staining alone. As measurements of dye volumes by densitometry have shown, double staining of two-dimensional separated pancreatic proteins is up to twenty times more sensitive than the usual Coomassie Brilliant Blue staining.

INTRODUCTION

Two-dimensional (2D) isoelectric focusing/sodium dodecyl sulphate (SDS) gel electrophoresis is a powerful tool in separating and characterizing proteins from complex mixtures [1]. Scheele [2] and Jutley *et al.* [3] have previously reported on the application of this method to analyse the protein composition of pure human pancreatic juice.

Coomassie Brilliant Blue R-250 is the most popular protein stain for 2D gels. Its main drawback is, however, its relatively low sensitivity. When using Coomassie Blue staining for 2D-separated human pancreatic proteins, there are some problems with the detection of basic proteins, such as proelastases and prophospholipase, glycoproteins and minor proteins present in pancreatic juice. Scheele [4] used the more sensitive silver staining to compare the protein patterns of pancreatic juice in normal subjects and patients with chronic pancreatitis. These studies demonstrated additional minor proteins not detectable in Coomassie-

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stained gels in patients with pancreatic disease. However, the application of the silver staining method also has some disadvantages with regard to spot discrimination; in addition, a number of proteins fail to stain at all [5].

To overcome these problems, our study aimed at improving the detection sensitivity of 2D-separated exocrine pancreatic proteins by means of consecutive staining with Coomassie Brilliant Blue and silver.

EXPERIMENTAL

Biological samples

Pure pancreatic juice was obtained from five patients by selective endoscopic cannulation of the main pancreatic duct, after stimulation with pancreozymin as described previously [6]. Only pancreatic juice without tryptic and chymotryptic activity was analysed.

Chemicals

Acrylamide (four-gold crystallized), N,N′-methylenebisacrylamide, N,N,N′,N′-tetramethylethylenediamine, ammonium persulphate, Servalyt 2–11, protein test mixture No. 9, Coomassie Brilliant Blue R-250 and G-250 were obtained from Serva (Heidelberg, F.R.G.). Trichloroacetic acid and sulphosalicylic acid (pro anal.) were purchased from Merck (Darmstadt, F.R.G.). All other chemicals were of analytical grade. Water was bidistilled and deionized.

Two-dimensional electrophoresis

Pancreatic proteins were separated by 2D electrophoresis using a Multiphor 2175 system from LKB (Bromma, Sweden): in the first dimension, urea isoelectric focusing (pH gradient 2–11, Servalyt; 5% T, 3% C polyacrylamide gel); in the second dimension, SDS gel electrophoresis in a Laemmli buffer system (10% T, 3% C, 0.1% SDS; 0.025 *M* Tris–0.192 *M* glycine at pH 8.3).

Staining procedures

Coomassie staining. Following SDS gel electrophoresis, gels were fixed in trichloroacetic acid (200 g/l) for 1 h; they were then stained with Coomassie Blue R-250 (1.5 g/l) in methanol–acetic acid–water (45:10:45, v/v) containing copper sulphate (5 g/l) for 1 h, and afterwards destained with a mixture of the same solvents (25:10:65) until a clear background was obtained.

Combined Coomassie G-250/silver staining. After SDS electrophoresis, double staining was performed according to De Moreno *et al.* [7], with some modifications. After fixing for 20 min using trichloroacetic acid (100 g/l)–sulphosalicylic acid (50 g/l), gels were stained for 15 min with Coomassie Blue G-250 (25 g/l) in a solution containing trichloroacetic acid (100 g/l) and methanol (50%, v/v). Destaining was performed in trichloroacetic acid (50 g/l) in three 10-min intervals. The gels were then washed twice in methanol–acetic acid–water (40:10:50, v/v)

and three times in ethanol–acetic acid–water (10:5:85, v/v) for 10 min. Then they were immersed for 5 min in potassium dichromate (0.0034 *M*)–nitric acid (0.0032 *M*) and washed three times in deionized water for 5 min. After a 20-min treatment with silver nitrate (0.012 *M*) gels were washed for 5 min with deionized water and developed in sodium carbonate (0.28 *M*) containing formaldehyde (0.5 ml/l), with one change of the solution after 5 min. Development was stopped with acetic acid (50 g/l) for 5 min, when a slightly yellowish background appeared. After two washing steps with deionized water the gels were immersed in water or dried. All steps were performed with gentle shaking at room temperature.

Gel scanning

Densitometric scans were carried out using an UltroScan XL laser densitometer from LKB (Bromma, Sweden) connected to an Olivetti personal computer M 24 (Ivrea, Italy) with the LKB gelscan software.

RESULTS AND DISCUSSION

Coomassie Brilliant Blue staining visualized nearly twenty spots of human pancreatic juice on the 2D gel (see Fig. 1). The pancreatic protein pattern found is similar to that reported by Scheele [2] and Jutley *et al.* [3]. Fourteen proteins were identified and characterized as digestive enzymes or proenzymes and glycoproteins, using substrate-specific detection methods as described elsewhere [2,8].

Pancreozymin-stimulated pancreatic juice from normal subjects was found to contain two forms of procarboxypeptidase A and trypsinogen, one form each of procarboxypeptidase B, α-amylase, lipase, chymotrypsinogen, trypsinogen-like zymogen and four glycoproteins. As seen in Fig. 1, it is difficult to demonstrate the presence of basic proteins such as proelastase 2 (IP 9.4, double band) and prophospholipase (IP 8.9) and some weakly acidic proteins (IP range 5.9–6.5) on the Coomassie Blue-stained 2D gels. The main drawback of this commonly used Coomassie Blue stain is its relatively low sensitivity.

Silver stains can detect electrophoretically separated proteins with up to 200 times more sensitivity than the Coomassie dye [9]. Scheele [4] reported the use of a diamine silver stain for the detection of proteins of pancreatic secretion with a low protein content. Similarly, Jutley *et al.* [3] used silver staining of pancreatic proteins after 2D electrophoresis, but they did not give any information about their staining method.

We also applied a silver staining procedure according to the method described by Merril *et al.* [9], with some modifications of the fixing step. We often observed that some proteins partially disappeared from the 2D gel when fixed with methanol–acetic acid. For this reason fixation was attempted with trichloroacetic acid (200 g/l), but a significant dark background of the gel was observed. A satisfactory reduction in background was obtained with a fixation mixture of trichloroacetic acid (100 g/l) and sulphosalicyclic acid (50 g/l).

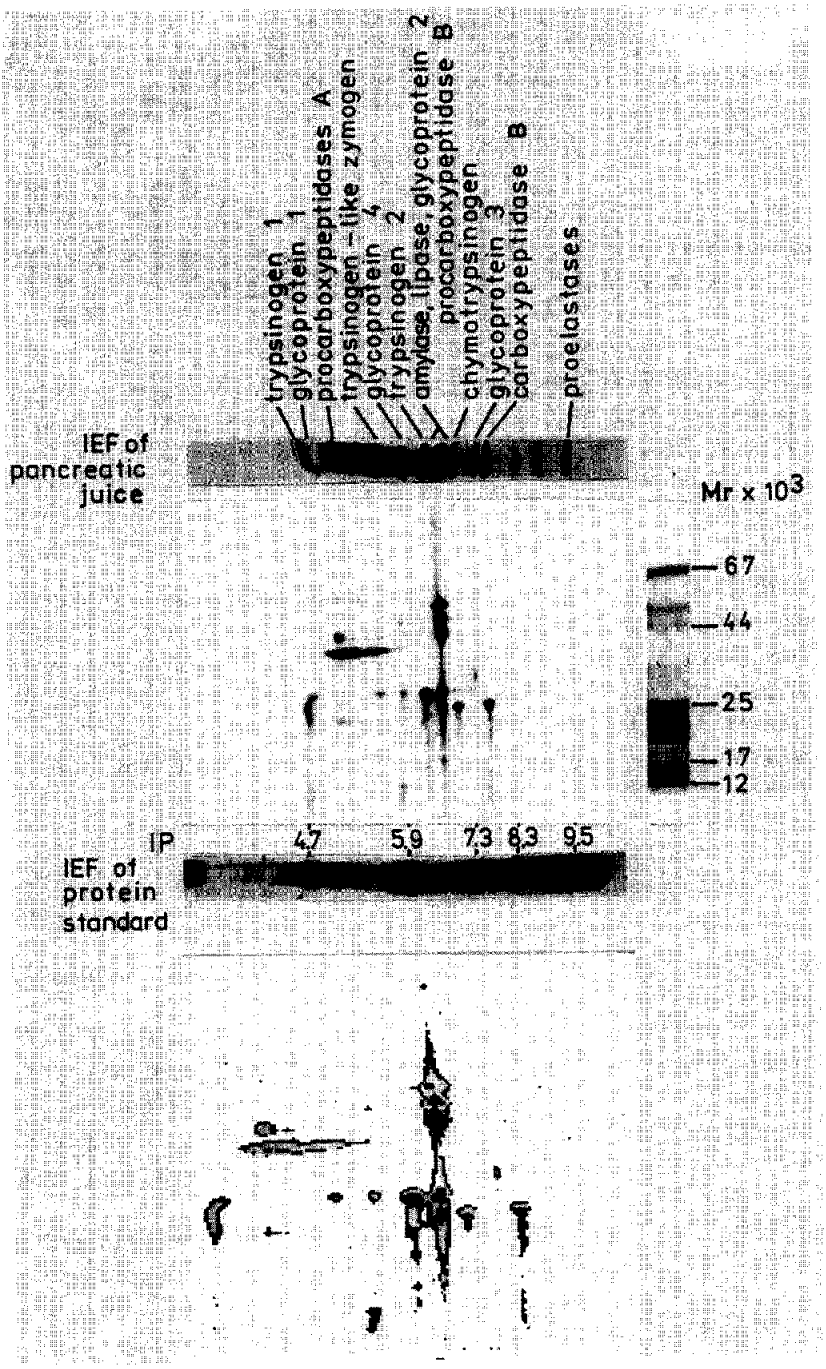

Fig. 1. 2D electrophoretic protein patterns of a subject without pancreatic disease: (upper) Coomassie Blue R-250 staining; (lower) corresponding densitometric scan.

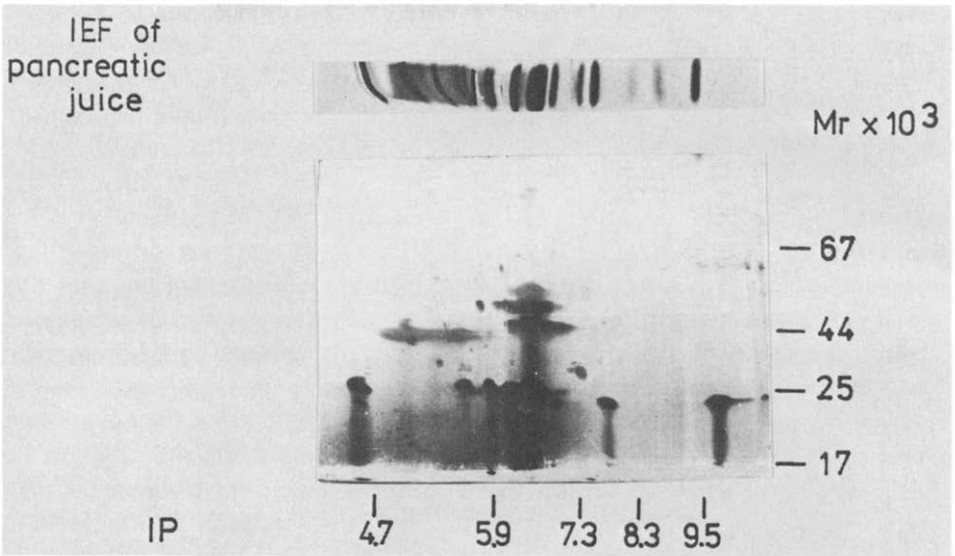

Fig. 2. Silver staining of the 2D electrophoretic pancreatic protein pattern. Pure pancreatic juice was from the same specimen as shown in Fig. 1. Identical protein amounts were loaded.

Using silver staining, an evident enhancement in sensitivity was observed (see Fig. 2). Proelastase 2 was clearly detected, and the number of unidentified proteins further increased, but some major proteins appeared smeared and a marked loss in spot discrimination was observed.

Another problem of silver staining was the different staining sensitivity of distinct proteins. Two glycoproteins (numbered glycoproteins 1 and 4 in Fig. 1) failed to stain with silver, whereas in the results reported by Jutley *et al.* [3] the presence of glycoprotein 1 was confirmed.

Owing to the unsatisfactory spot discrimination and the different staining sensitivity of distinct proteins, silver staining of 2D maps of pancreatic proteins appears unsuitable.

In order to overcome these problems, we applied a double-staining method with Coomassie Blue and silver for a highly sensitive detection of 2D-separated exocrine pancreatic proteins. The cause of the increased sensitivity of this combined procedure may be site-enhanced silver nucleation caused by the sulphonic acid dye [7].

Fig. 3 contains photographs of the double-stained 2D gel and the corresponding densitometric scan from the same specimen of pure pancreatic juice as shown in Fig. 1 (identical protein amounts were loaded). A comparison of the 2D electrophoretical patterns of exocrine pancreatic proteins obtained by the two different staining procedures indicates an enhanced detection sensitivity and a satisfactory spot discrimination with the double-staining procedure. As shown in

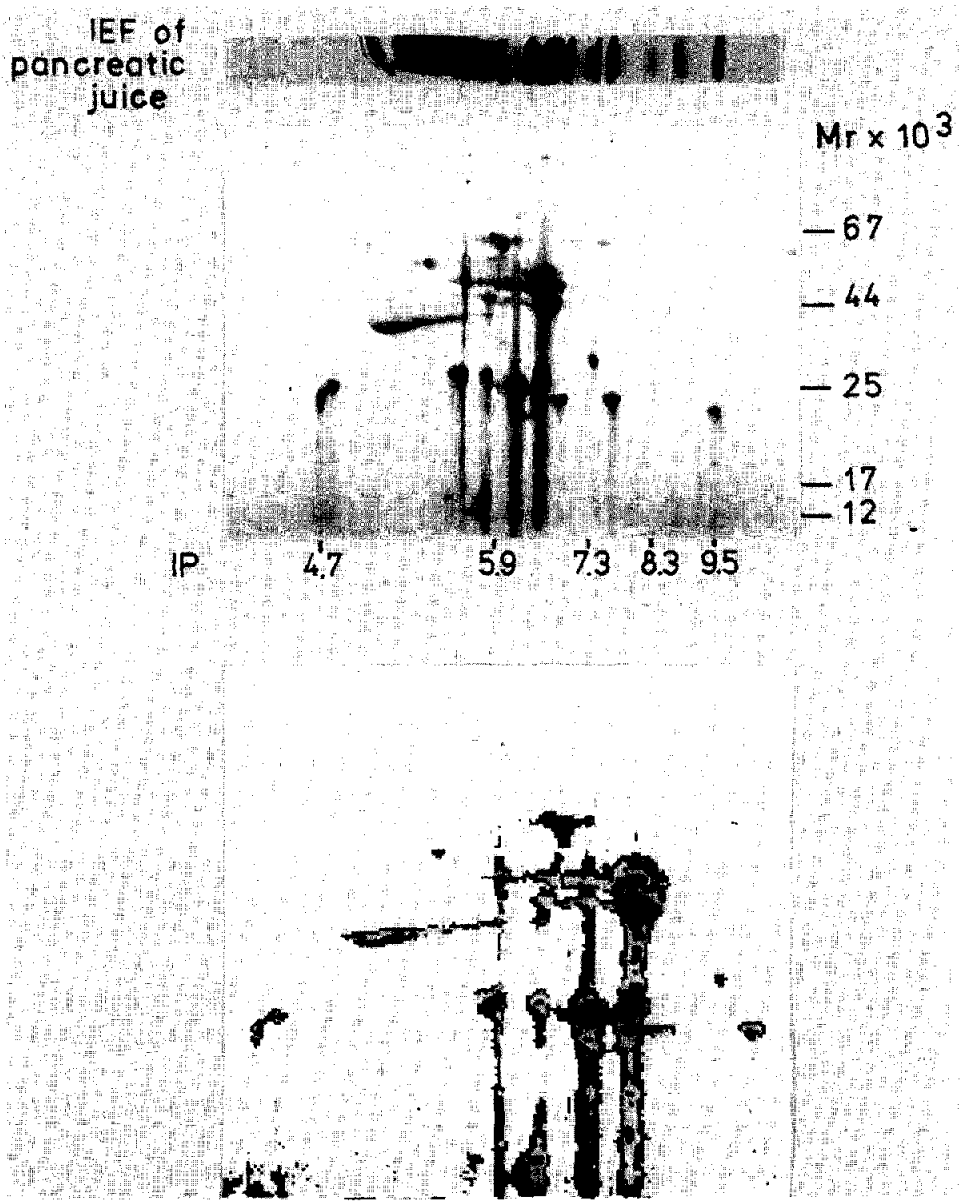

Fig. 3. Coomassie Blue/silver double-stained 2D electrophoretical pancreatic protein pattern (above) and the corresponding densitometric scan (below). The sample of pancreatic juice was the same as used for Figs. 1 and 2.

Figs. 1 and 3, the differences between the two staining methods are clearer when the plots of the corresponding densitometric scans are compared. A number of new or intensified spots, particularly in the IP range 5.9–6.5, are present.

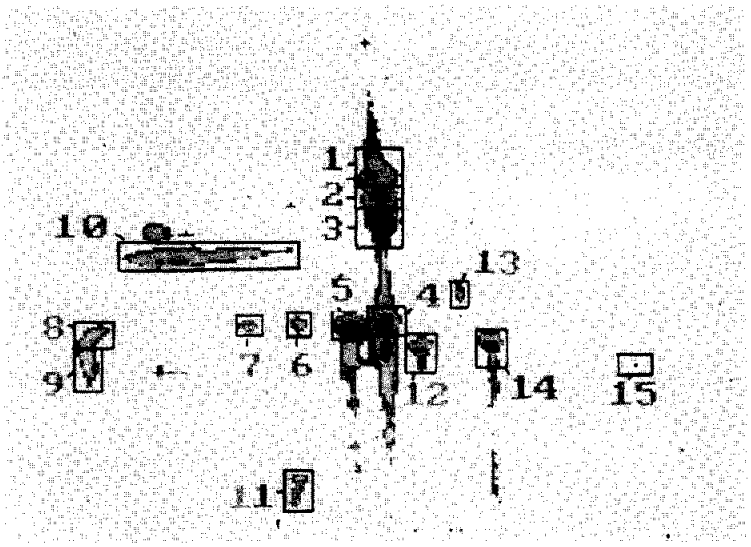

Fig. 4. Plot of a densitometric scan of a 2D protein pattern. The labelled protein spots (1–15) were included in a computer analysis to calculate the dye volumes.

TABLE I

DYE VOLUMES OF PANCREATIC PROTEINS: COMPARISON OF COOMASSIE BLUE AND DOUBLE-STAINING TECHNIQUES

The dye volume of a protein spot was calculated by the computer program from the mean dye area of a spot and the height of absorbance at 632 nm.

No.	Protein	Relation of dye volumes of distinct proteins to the dye volume of amylase	
		Coomassie staining	Double staining
1	α-Amylase	1.00	1.00
2	Lipase	2.49	4.30
3	Procarboxypeptidase B1	13.91	33.20
4	Glycoprotein 2	3.30	20.20
5	Trypsinogen 2	5.04	13.20
6	Trypsinogen-like zymogen	0.55	5.20
7	Not identified	0.57	21.30
8	Trypsinogen 1	2.34	5.10
9	Not identified	1.70	4.50
10	Procarboxypeptidase A1	13.30	71.30
11	Glycoprotein 4	0.45	4.40
12	Chymotrypsinogen	2.02	6.02
13	Glycoprotein 3	2.40	12.10
14	Carboxypeptidase B	0.42	19.00
15	Proelastase 2	Not detectable	0.9

In the double-stained gel, the horizontal bands across the entire 2D gel near the molecular mass region of 67 000 (see Fig. 3) have been attributed to 2-mercaptoethanol in the equilibrium buffer. The appearance of small vertical streaks is also due to the presence of this reagent. This problem has previously been encountered in silver and double stainings used in 2D electrophoresis [1].

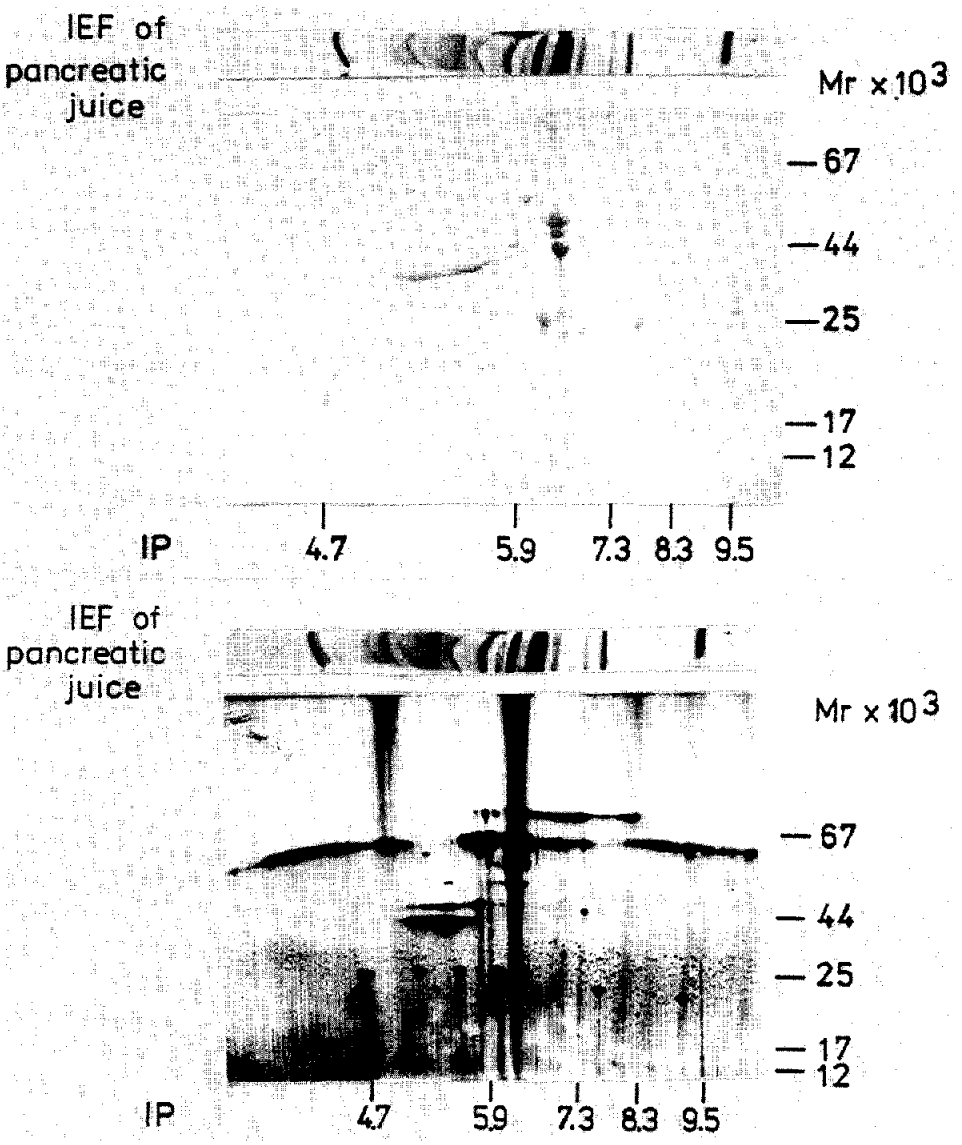

Fig. 5. 2D electrophoretic protein patterns of a patient with chronic pancreatitis: (upper) Coomassie Blue R-250 staining; (lower) combined Coomassic Blue/silver staining.

The superiority of the double-staining technique becomes even more evident when the dye volumes of the spots determined by 2D gel scanning and computer analysis are considered. The volumes of dye of amylase (spot No. 1 in Fig. 4) were set to 1. Amylase was chosen as a reference because of its very good detectability with both of the investigated staining methods. Furthermore, amylase is one of the pancreatic juice proteins most resistant to autodigestion. The results of calculations are summarized in Table I. The running numbers of proteins given in Table I correspond to those indicated in Fig. 4, which shows the plot of a scanned gel pattern. Protein spots included in the dye volume calculation are labelled with numbers from 1 to 15. The values of dye volumes of the individual spots using the double-staining method, are from two (Table I, No. 2 and 8) to twenty times (Table I, No. 7 and 14) greater than with common Coomassie Blue stain.

In addition, the double-staining method seems advantageous in analysing pure pancreatic juice from patients with chronic pancreatitis, as our preliminary investigations of such a specimen indicated, albeit tentatively (Fig. 5).

CONCLUSIONS

2D electrophoresis with combined Coomassie Blue/silver staining offers an extremely sensitive and specific technique to study the heterogeneity of human pancreatic proteins. Significant advantages in terms of sensitivity and discrimination power over the separate Coomassie Blue and silver stains have been found.

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