SPECIFIC ISOLATION OF CYSTEINE PEPTIDES BY COVALENT CHROMATOGRAPHY ON THIOL AGAROSE DERIVATIVES

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1. Introduction

Methods based on such parameters as size, charge and degree of hydrophobicity are used routinely for the fractionation of peptide mixtures. Here we describe a method for the specific isolation of thiol-containing peptides. The principle is to form mixed disulphides between the cysteinyl thiols of the peptides and a thiol-containing insoluble polymer, thereby immobilizing the thiol peptides on the matrix. After washing away the unbound peptides the cysteinyl peptides are released by reduction of the peptide-polymer disulphides. The formation of disulphides is favoured by activation of one of the thiols with 2,2′-dipyridyl disulphide to form the mixed disulphide derivative.

Recently another method based on the same reaction was described [1] wherein cysteinyl-containing proteins were immobilized to activated thiol agarose followed by proteolytic digestion, elution of non-thiol peptides and removal of the cysteinyl peptides from the polymer matrix by reduction.

Earlier some mercurial derivatives of agarose have been used for the same purpose. Thiol peptides from lombricine kinase of *Lumbricus terrestris* were isolated by immobilization on and desorption from a mercury agarose derivative [2]. Two SH-containing peptides from polypeptide chain elongation factor Tu were isolated on *p*-chloromercuribenzoate–agarose [3] by using a similar technique.

2. Materials and methods

Bovine serum albumin and pancreatic ribonuclease were obtained from Sigma Chem. Co. Ribonuclease was reduced with 2-mercaptoethanol in 8 M urea according to Crestfield et al. [4]. The thiol polymers used were glutathione–Sepharose 4B prepared by reduction of activated thiol-agarose obtained from Pharmacia, Uppsala, Sweden or the thiol-agarose generated by reduction the *S*-sulfo alkyl derivative prepared by treatment of epoxy-activated Sepharose 2B with sodium thiosulphate [5]. The polymers were reduced with 25 mM dithioerythreitol (Sigma) at pH 8.7 for 30 min immediately prior to use.

Thiol contents were determined with 2,2′-dipyridyl disulphide (Aldrich-Europe, Beerse, Belgium) according to the method of Grassetti and Murray [6] as modified for determinations in gels by Brocklehurst et al. [7]. The thiol contents of the two polymers used were 40 and 32 μequiv./g dry weight, respectively. A modification of the method was used to prepare the mixed disulphides between thiopyridine and the thiols of serum albumin and reduced ribonuclease. In the case of ribonuclease the reduced protein was applied to a Sephadex G-25 column (3.2 × 7 cm) equilibrated with 1 mM, 2,2′-dipyridyl disulphide in 0.2 M formic acid–acetic acid, pH 2.0 and elution was done with the same buffer at 25 ml/h. The protein fraction was freed of excess reagents by passing the material through another Sephadex G-25 column in a 0.2 M formic acid–acetic acid buffer, pH 2.0.

Serum albumin and reduced ribonuclease thus activated were digested with pepsin. Pepsin (Sigma) was dissolved in water and added to the solutions of proteins in a 1:40 (w/w) ratio and digestion was performed at pH 2.0 for 6 h at 30°C.

The peptic digest was mixed with thiol-agarose in 0.2 M formate–acetate buffer, pH 2.0. The production

of thiopyridone was followed by spectrophotometry. When no further change in absorption at 343 nm was seen (6 h) the gel was washed on a glass filter. The two first washes were collected and lyophilized. After thorough washing, the gel was packed into a column (1 × 10 or 3.2 × 4 cm) and washed with 3 vol. 0.1 M ammonia–ammonium acetate, pH 8.5.

Peptides that had formed disulphides with the thiol gel were then removed with 50 mM 2-mercaptoethanol in the same buffer. The flow rate was 10 ml/h and three column volumes of eluate were collected and lyophilized.

The peptide fractions were analyzed by paper electrophoresis. Samples were applied on Whatman 3 MM paper sheets and run in a Gilson Medical Electronics high voltage paper electrophoresis apparatus. The buffers used were 0.041 M pyridine/0.58 M acetic acid, pH 3.50, or 1.24 M pyridine/0.069 M acetic acid, pH 6.46. The papers were stained with ninhydrin (0.2% solution in ethanol/acetic acid/collidine, 60:20:8, v/v/v) [8]. In the case of serum albumin a guide strip was stained and from a parallel strip, the locations corresponding to the spots were cut out and eluted from the paper with 10% acetic acid.

The peptide fractions from ribonuclease were also analyzed by diagonal electrophoresis to distinguish cysteine-containing peptides from the others [9]. The paper strip containing the separated peptides was cut out and treated with performic acid vapor, dried carefully, then sewn onto a new paper sheet. Electrophoretic separation was then performed in the perpendicular direction under conditions identical to those used in the first.

Amino acid analysis was done with 6 N HCl at 100°C for 24 h in thoroughly evacuated sealed tubes. The hydrolysates were analyzed on a Durrum D-500 amino acid analyzer [10].

3. Results and discussion

The thiol group of bovine serum albumin has been shown to react readily with pyridyl disulphide under a variety of conditions [10]. Serum albumin (1 μmol) was thus mixed with 10 ml pyridyl disulphide (1 mM) in 0.2 formate–acetate pH 2.0 or 0.2 M ammonium acetate, pH 8.0, for 30 min at 25°C after which no further change in A_{343} was observed. The amount of 2-thiopyridone liberated was 0.67 μmol. The excess of reagents was removed by passing the material through a Sephadex G-25 column as described above.

The yield for the immobilisation of activated cysteinyl peptides from the protein digests was calculated from the concentration of liberated 2-thiopyridone in the gel suspension: 0.6 μmol (90%) was detected for serum albumin and in the case of ribonuclease about 75% of the activated cysteines had reacted with the thiol agarose. The immobilisation of activated thiol peptides from mixtures containing disulphide peptides should be performed at acidic pH. Preliminary experiments with the serum albumin peptide mixture immobilized at pH 8.7 showed extra peptide bands upon analysis by paper electrophoresis of the material, eluted with mercaptoethanol. This was interpreted to mean that the disulphide-containing peptides had reacted with thiols or rather thiolate ions of the gel matrix.

As reducing agents 2-mercaptoethanol (50 mM) or dithioerythritol (20 mM) were used but the former was preferred, since it evaporated to a large extent on lyophilisation and did not interfere with the peptide analyses. Trace amounts of mercaptoethanol remaining prevented reoxidation of cysteinyl thiols.

Analyses by paper electrophoresis of the albumin peptide material eluted with the reducing agent is shown in fig.1. The main peptide fraction from the albumin digest corresponds well in mobility with earlier preparations of the peptic cysteinyl peptide from serum albumin [1]. At the location corresponding to glutathione in the electrophoretic run one spot was observed with the material prepared by using glutathionyl-agarose. This is in agreement with earlier findings [1], where glutathione was suggested to be liberated from the gel upon addition of mercaptoethanol. Consequently the spot was missing in the material prepared from thioalkyl-agarose. The recovery of peptide was 70%.

Amino acid analysis of this peptide fraction showed the same amino acid composition as previously reported [1,11] i.e., Leu, Pro, Glx_3, Asx, Cys, and Phe. These are the residues 31–38 around the single thiol group in serum albumin. The peptide obtained from serum albumin using thiols activated at pH 2 was the same as that obtained from activation at pH 8, as seen in fig.1, indicating that the increased reactivity of the albumin thiol at acidic pH was not due to the

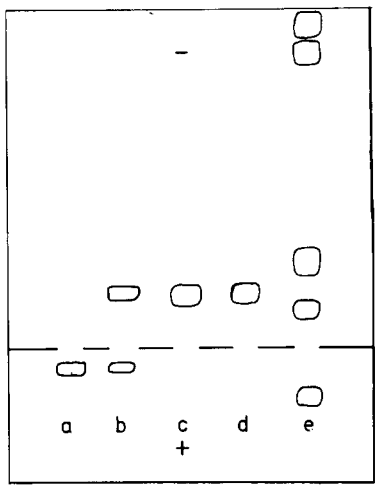

Fig.1. Paper electrophoresis of the peptide fraction of digested bovine serum albumin prepared by covalent chromatography on thiol-agarose. Peptides were separated at pH 3.50 for 30 min at 3000 V. (a) Reduced glutathione (25 nmol). (b) Peptide fraction from albumin eluted with reducing agent from glutathione agarose. (c, d) The same fraction prepared from thioalkyl-agarose and bovine serum albumin activated with pyridyl disulphide at pH 2.0 and 8.0 respectively. (e) Mixture of amino acids (20 nmol of each) i.e., Lys, Arg, His, neutrals, Glu, and Asp.

appearance of another, more reactive, thiol [12].

Ribonuclease with modified cysteinyl residues, prepared as described above, was analyzed by addition of dithioerythritol (20 mM) to a sample of the protein at pH 8.0 in 0.2 M ammonium acetate. Liberated thiopyridone was determined by spectrophotometry. The ribonuclease concentration was estimated from the ultraviolet spectrum after correction for the contribution from the thiopyridone absorption. The amounts of thiopyridone liberated were 6.7–7.2 equiv./mol ribonuclease, indicating a high degree of modification of the cysteine residues.

The thiol peptides of ribonuclease were characterized by the change in electrophoretic mobility upon oxidation of the cysteine thiols to sulphonic acid. The diagonal maps shown in figs.2 and 3 represent the fraction of the peptic digest that coupled to thiol agarose and the one that did not, respectively. The maps show that the peptides in the material, eluted with reducing agents, increase their mobility towards the anode. The increase seems equal for all separated spots, except that corresponding to glutathione, as

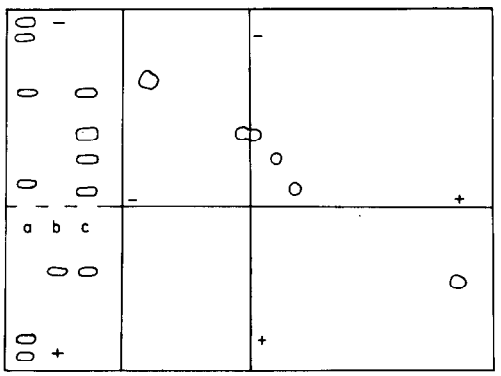

Fig.2. Diagonal paper electrophoresis of peptic peptides from ribonuclease fractionated by reversible immobilisation to glutathionyl-agarose. The electrophoreses were performed at pH 6.46 with 3000 V for 30 min. (a) Reference amino acids (see fig.1 e). (b) Reduced glutathione (25 nmol). (c) Ribonuclease peptides. Performic acid oxidation was performed on the paper between the two duplicate electrophoretic runs.

would be expected from the oxidation of one cysteine to cysteic acid. The residual peptides from the immobilisation to the thiol-agarose showed no increase in mobility, as seen in fig.3. The peptide mixture from ribonuclease seems therefore to be separated into a thiol-containing and one non-thiol peptide fraction.

As shown in fig.2 peptide material with an electrophoretic mobility corresponding to reduced

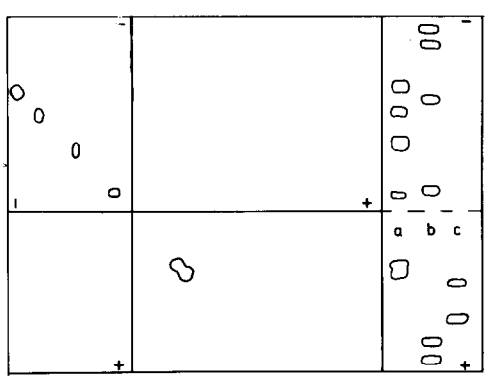

Fig.3. Diagonal paper electrophoresis of ribonuclease peptides that did not couple to the glutathionyl agarose. Conditions as described in fig.2. (a) Ribonuclease peptides. (b) Reference amino acids. (c) Reduced and oxidized glutathione (25 nmol of each). The paper was treated with performic acid between the two directions of electrophoresis.

glutathione was present in the peptide mixture that was eluted with reducing agent from glutathionyl-agarose. This is in accordance with earlier observations [1]. The use of thioalkyl-agarose therefore seems preferable, since the glutathione 'leakage' can interfere with further separation and analysis work.

The present application of covalent chromatography for isolation of thiol-containing peptide in proteins, extends the use to polythiol proteins such as reduced disulphide-containing proteins. Activation of cysteine thiols prior to proteolytic digestion also prevents intramolecular thiol–disulphide exchange reactions that might occur during the proteolytic digestion of the unblocked thiol protein.

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