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AMINO ACID MICROANALYSIS OF PROTEINS EXTRACTED FROM SPOTS OF FIXED, STAINED, TWO-DIMENSIONAL GELS

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SUMMARY

A method for the extraction of proteins from fixed, stained, two-dimensional polyacrylamide gels and subsequent amino acid microanalysis was described. Human serum proteins were separated by two-dimensional electrophoresis, the stained spots were punched out and the proteins in each piece of gel were extracted with 0.1 *M* sodium hydroxide–2% thiodiglycol. The extracted proteins were hydrolysed and applied to an amino acid analyser equipped with a fluorimeter for detection of the reaction products with *o*-phthaldialdehyde. By reducing the amount of background contaminants, amino acid analysis of 1 μg or less of extracted proteins became possible. The amino acid composition of the proteins was compared with reported compositions of serum proteins by calculating correlation coefficients, which we designated "similarity indices of amino acid composition". These indices were useful for the identification of the extracted proteins.

INTRODUCTION

In previous reports, we described a technique of two-dimensional electrophoresis in the absence of denaturing agents[1,2] and its applications to the analysis of human immunoglobulin myeloma proteins[3] and human α-amylase isoenzymes[4]. With this technique, p*I* values and approximate molecular weights of 128 human plasma protein spots were determined and about 80 spots were identified as known plasma proteins[5].

During the course of the identifications of plasma proteins, we intended to extract proteins from the fixed, stained, slab gels and to analyse their amino acid compositions. Extraction of proteins from polyacrylamide gel and subsequent amino acid analysis have been reported[6–9]. However, we found that these existing methods were not sufficiently sensitive for our purpose[6,7] or had complicated extraction steps[8,9].

We report here a simple method for the amino acid microanalysis of proteins extracted from fixed, stained, two-dimensional polyacrylamide gels. The method is useful for the subsequent analysis of proteins separated by two-dimensional electrophoresis.

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EXPERIMENTAL

Materials

o-Phthaldialdehyde (OPA) (analytical-reagent grade), 6 *M* hydrochloric acid for amino acid sequence analysis and other reagents for amino acid analysis were obtained from Wako (Tokyo, Japan). Amino acid standards were obtained from Takara Kosan (Tokyo, Japan). Ampholines, pH 3.5–10 and 3.5–5, were purchased from LKB (Bromma, Sweden). Other reagents for electrophoresis were of special grade or better from Wako Nakarai Chemicals (Kyoto, Japan). Water for the preparation of acidic and basic buffers for amino acid analysis and for solutions used in the extraction procedure (7% acetic acid and 0.1 *M* sodium hydroxide–2% thiodiglycol) were prepared with a system consisting of an ion-exchange column and a glass distillation apparatus and was collected directly from the distillation condenser.

Two-dimensional electrophoresis

Human serum (50 μl) was subjected to two-dimensional electrophoresis as described earlier[1,2]. The second-dimension slab gel was a 4–21% (or 4–17%) linear acrylamide gradient gel and was 16 cm wide, 12 cm high and 0.4 cm thick. The gels were stained with 0.025% Coomassie Brilliant blue R-250–50% (v/v) methanol–7% (v/v) acetic acid overnight and destained in 7% (v/v) acetic acid at 80°C for 4 h and in two changes of 7% (v/v) acetic acid at room temperature for 2 days.

Extraction of proteins from polyacrylamide gel matrix

A two-dimensional electrophoretic slab gel that had been stained and destained was placed in a plastic container, 1 l of 7% (v/v) acetic acid was added to the container and the gel was allowed to stand for 1 h in the container with a lid. Then the acetic acid solution was removed by decanting the container (the gel was held using a polyethylene disposable glove). Stained spots on the gel were punched out with a stainless-steel tube (inner diameter 2 mm, commonly used in immunoelectrophoresis for making sampling holes on agar gel plates). The piece of gel was pushed out with a thin glass rod into a Pyrex test-tube (100 × 12 mm I.D., calcined at 500°C for 3 h), 0.2 ml of freshly prepared 0.1 *M* sodium hydroxide–2% thiodiglycol was added with a glass micropipette and the tube was allowed to stand for 10 min at 40°C, then the solution was removed with the micropipette and discarded. A further 0.2 ml of 0.1 *M* sodium hydroxide–2% thiodiglycol was added to the test-tube, the mixture was allowed to stand for 10 min at 40°C, then the solution was transferred into another calcined hydrolysis tube (100 × 12 mm I.D. or 55 × 4 mm I.D.).

Preparation of samples for amino acid analysis

The solution was freeze-dried and 0.5 ml of 6 *M* hydrochloric acid was added to the test-tube. The tube was evacuated for 5 min and sealed, and hydrolysis was performed at 110°C for 24 h. The tube was then opened and 150 μl of 0.1 *M* hydrochloric acid–1% thiodiglycol were added. The tube was agitated on a mixer for 10 sec and 100 μl of the solution were subjected to amino acid analysis.

Amino acid analysis

Analyses were performed with a Nikon–Rank Hilger Chromaspek J-180

amino acid analyser (Rank Precision Industries, Westwood, Great Britain), which was equipped with a column (350 mm × 3.0 mm I.D.) packed with divinylbenzene–styrene cation-exchange resin (6 ± 1 μm), and a fluorimeter for detection of OPA reaction product. A schematic diagram of the modified flow system of the amino analyser is shown in Fig. 1. The buffers for elution and for fluorescence development were modified as follows. The acid buffer contained 42.0 g of citric acid, 25.4 g of lithium chloride, 14.0 ml of 10% Brij 35, 0.8 ml of *n*-caproic acid, 2.5 ml of thiodiglycol and 4 l of water. The basic buffer contained 42.0 g of citric acid, 50.4 g of lithium hydroxide monohydrate, 35.2 g of boric acid, 14.0 ml of 10% Brij 35, 0.4 ml of *n*-caproic acid and 4 l of water. The gradient elution was controlled with a programmer.

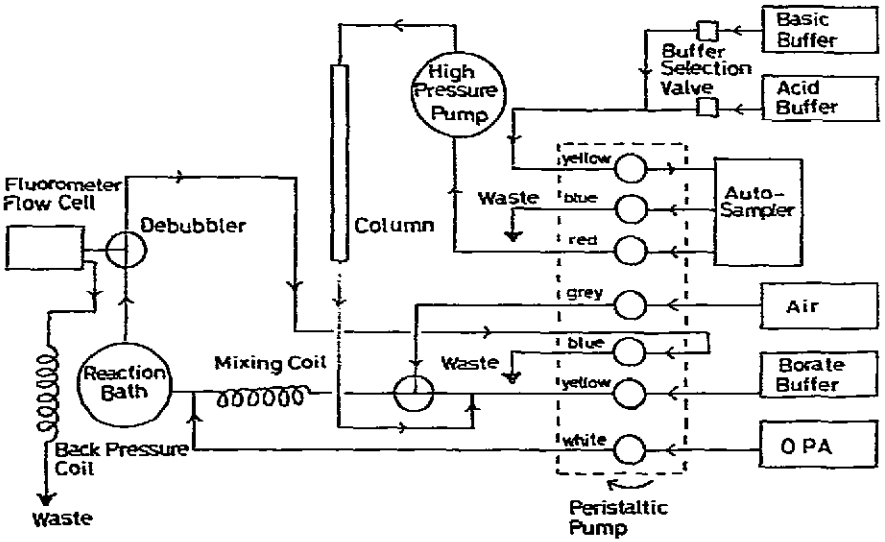


Fig. 1. Schematic diagram of the amino acid analyser equipped with a fluorimeter for detection of reaction product.

Samples for analysis were introduced into the column via a filling device adjusted to accomodate 100 μl of sample. The column temperature was controlled from 40 to 96°C during one analysis. Borate buffer, which contained 370.8 g of boric acid, 10 *M* potassium hydroxide solution added to give pH 10.5 and made up to 4 l with water, was mixed with the column effluent, passed through a mixing coil (50 cm × 1.0 mm I.D.; coil radius 6 mm), mixed with OPA solution which contained 3.2 g of OPA, 80 ml of ethanol, 8 ml of 2-mercaptoethanol, 8 ml of 15% sodium hypochlorite and 4 l of water, and then passed through a reaction coil at 44°C. The effluent was passed into a fluorimeter, with an excitation wavelength of 350 nm and an emission wavelength of 450 nm. Amino acids were quantitated by measuring their peak areas with a Shimadzu Chromatopak C-E1 B integrator (Shimadzu, Kyoto, Japan).

Calculation of correlation coefficients

The amino acid composition of the protein in a piece of stained gel was calculated by subtracting the amounts of amino acids in a piece of background gel from those of the piece of stained gel. The correlation coefficients between the measured

amino acid composition of an extracted protein and the reported compositions of serum proteins, which we called "similarity indices" (γ), were calculated according to the equation

$$\gamma = \frac{n\Sigma x_i y_i - \Sigma x_i \Sigma y_i}{\sqrt{[n\Sigma x_i^2 - (\Sigma x_i)^2]\,[n\Sigma y_i^2 - (\Sigma y_i)^2]}}$$

where x_i represents the measured content of an amino acid (i), y_i the content in the literature of the same amino acid (i) and n is the number of (x_i, y_i) pairs (number of amino acid species) used for the calculation. The calculated similarity indices were sorted with a microcomputer NEC PC-8001 (Nippon Electric, Tokyo, Japan). The amino acids used for the calculation were Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Ile, Leu, Tyr, Phe, Lys, His and Arg. The serum proteins, the amino acid compositions of which were used to calculate the correlation coefficients, were: C1 inactivator[10], prealbumin[11], transferrin[12], albumin[13], C1q complement[14], C3 complement[15], Gc-globulin[16], thyroxin-binding globulin[17], haemopexin[16], α_1-antitrypsin[18], α_1-antichymotrypsin[18], inter-α-trypsin inhibitor[18], anti-thrombin[19], α_2-macroglobulin[20], proteinase inhibitor[20], low-density lipoprotein (LDL)[21], apo-high-density lipoprotein[22], kininogen[23], ceruloplasmin[24], α_1-acid glycoprotein[25], α_1-glycoprotein easily precipitating[18], α_2-HS glycoprotein[18], histidine-rich α_2-glycoprotein[26], Ba-α_2-glycoprotein[27], α_2-β_1-glycoprotein 4S[28], β_1-glycoprotein[29], β_2-glycoprotein[18], fibrinogen[30], α_{2H}-globulin[31] and α_1-α_2-globulin urate binding[32].

RESULTS

Method of extraction

Before examining extraction methods, direct hydrolysis of the piece of gel that contained stained proteins was examined. However, amino acid analysis of the hydrolysate showed a large amount of ammonia derived from the hydrolysis of polyacrylamide gel, and the broad ammonia peak overlapped the peaks of lysine and arginine. Moreover, tailing of the ammonia peak enhanced the baseline fluorescence of the next analysis. As direct hydrolysis of the gel was not successful, we tried to extract stained proteins from the polyacrylamide gel matrix.

We chose extraction with 0.1 *M* sodium hydroxide–2% thiodiglycol as described under Experimental. Fig. 2 shows how the amount of extracted protein change on repeating the extraction cycle. Purified human albumin (2 mg) was subjected to two-dimensional electrophoresis and a gel punched from the albumin spot and that from background gel position were compared. The solution obtained after each extraction cycle was freeze-dried and hydrolysed and the hydrolysate was subjected to amino acid analysis. The protein content of the solution was calculated by adding the amounts of amino acids. In the first cycle of extraction, the pH of the extraction solution was about neutral, as the slab gel had been soaked in 7% (v/v) acetic acid. The amounts of proteins obtained in this extraction step from the albumin gel and the background gel were almost the same. In the second cycle of extraction, the amount of protein extracted from the albumin gel was about 8 times greater than that from the background gel. The pH of the extraction solution after the second extraction cycle was about 13. When the extraction cycle was repeated further, the

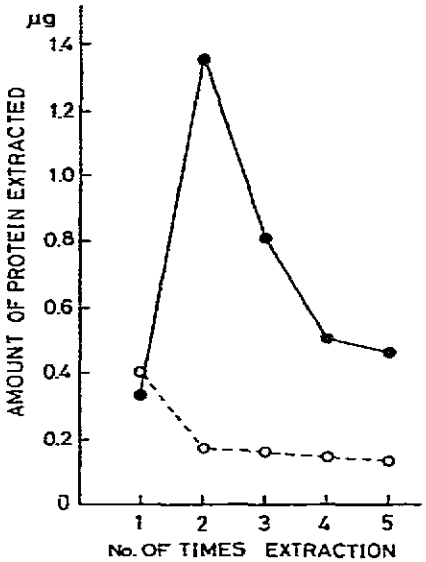


Fig. 2. Change in the amount of protein extracted on repeating the extraction cycle for a piece of gel punched out from the albumin spot (●) and for a piece of background gel (○).

recovery of protein from the albumin gel decreased and then the ratio of protein content of albumin gel extract to background gel extract decreased. Therefore, the first cycle was actually the step of washing the piece of gel and the second cycle was the step of protein extraction.

Amino acid microanalysis

Fig. 3 shows one of the two-dimensional polyacrylamide slab gels used to punch out gel pieces. The holes on the slab gel show the positions where the pieces of gel were punched out with a stainless-steel tube. The arrows show the positions where background gel pieces were taken out. Because the procedure is simple, extraction from 25 gel pieces could be done within 2 h.

Fig. 4 shows the relative peak heights of equimolar (250 pmol) amounts of amino acids, determined by the OPA method with a Nikon–Rank Hilger amino acid analyser. An integrator was used to calculate peak areas of amino acids and individual amino acids in amounts as small as 5 pmol could be determined.

Fig. 5 shows two examples of amino acid analysis of the gel extract. Fig. 5A and B show the elution patterns of amino acids of the extract from a piece of gel positioned at spot 80 (Fig. 3) and that extracted from a piece of background gel, respectively. The spot number corresponded to that in the "normalized map" of plasma proteins, which illustrated the standard distribution of the proteins on two-dimensional electrophoretic gels[5]. The amounts of amino acids found in the hydrolysate of the background gel extract were subtracted from those in the hydrolysate of the sample gel extract and the amino acid composition of the extracted protein was calculated.

Table I shows three examples of the amino acid compositions of extracted protein. The amino acid compositions of protein extracted from spots 80, 81 and 82

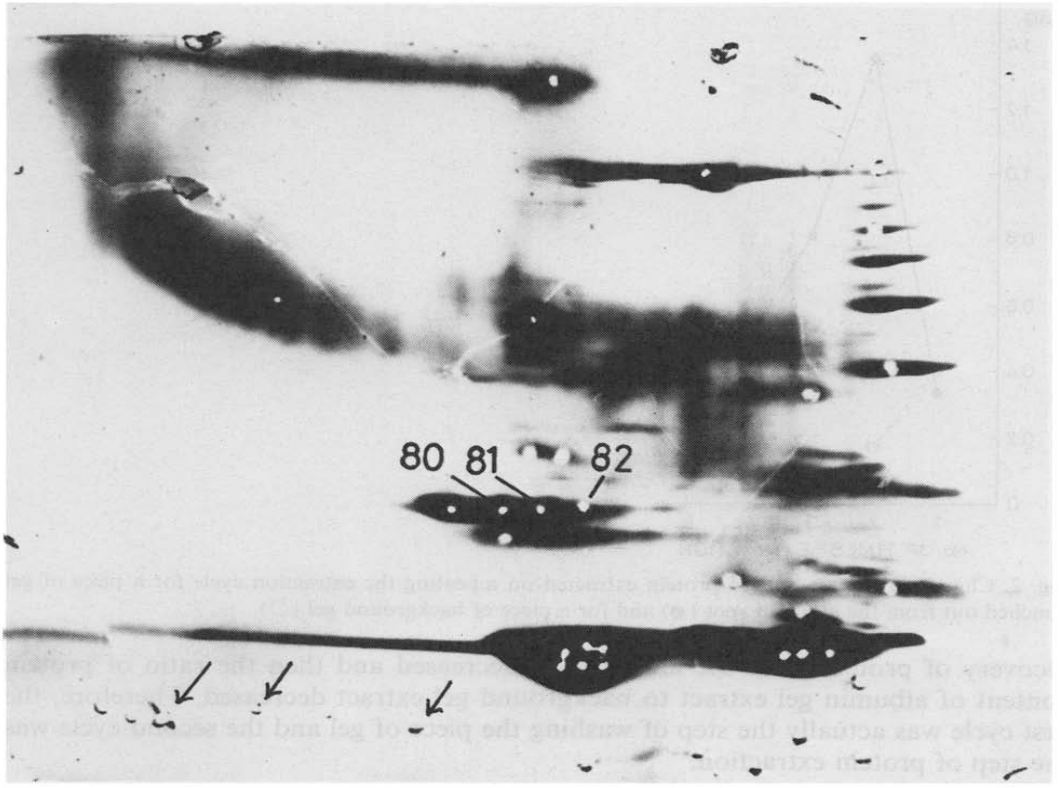
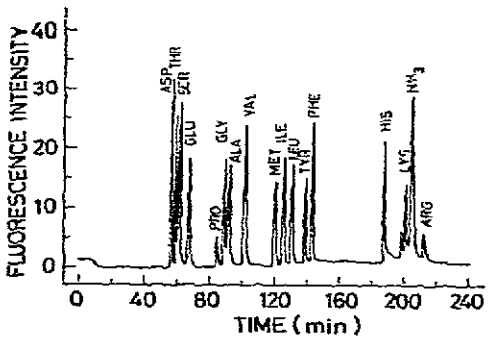


Fig. 3. Two-dimensional polyacrylamide slab gel used to punch out stained gel pieces. Spot numbers corresponded to those in the "normalized map" of human plasma proteins[5] and the amino acid compositions of the proteins extracted from the numbered spots are shown in Table I. The arrows show the positions where pieces of background gel were punched out.

resembled that of transferrin[12]. However, the contents of cysteine and methionine of the extracted proteins were much lower than the reported values, suggesting that these amino acids have been oxidized. The amounts of protein extracted were 0.92 μg from spot 80, 0.72 μg from spot 81 and 0.50 μg from spot 82. The considerable differences in the amounts of glycine and histdine in spot 82 from the reported values may be due to the relatively high background level of these amino acids (Fig. 5B). The maximal amount of protein extracted from one piece of gel was about 1.5 μg (from the albumin spot) and about 0.6–1.0 μg of protein was extracted from the pieces of gel positioned at the spots of major serum proteins (those which have serum concentrations exceeding 40 mg/dl).

The similarity indices of the amino acid composition of the extracted protein and the compositions of 30 human serum proteins were calculated using a microcomputer, in an attempt objectively to describe the "degree of similarity" between amino acid compositions. Table II shows some of the results of the calculation for the amino acid compositions of spots 80, 81 and 82 (Table I). Although the microcomputer was programmed to calculate similarity indices with 30 serum proteins simultaneously, only the highest and next to highest "similarity indices" are listed in the table in order



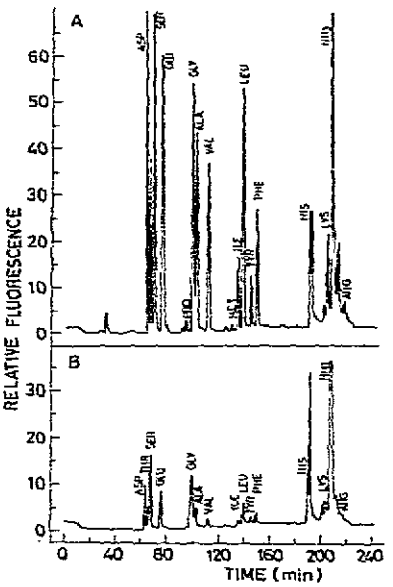


Fig. 4. Relative peak heights of equimolar (250 pmol) amounts of amino acids determined by the OPA method.

Fig. 5. Two examples of amino acid analysis of the gel extract. (A) Elution pattern of amino acids of the extract from a piece of gel positioned at spot 80 (Fig. 3); (B) elution pattern of background gel extract.

TABLE I

THREE EXAMPLES OF AMINO ACID COMPOSITIONS OF EXTRACTED PROTEINS

Extracts from spots 80, 81 and 82. The reported compositions of transferrin[12] is also listed. The reported number of alanine residues was used to compare the amino acid compositions.

Amino acid	*Protein extracted*			*Transferrin reported*[12]
	From spot 80 (0.92 μg)	*From spot 81 (0.72 μg)*	*From spot 82 (0.50 μg)*	
Asp	69.8	66.7	67.2	71
Thr	24.6	25.5	24.7	25
Ser	35.4	28.9	31.1	35
Glu	53.2	59.1	66.0	53
Pro	25.8	29.4	26.9	36
Gly	47.0	46.3	64.5	46
Ala	*51*	*51*	*51*	*51*
Cys/2	0	0	0	30
Val	39.4	40.9	39.2	40
Met	0	3.1	0	8
Ile	13.7	14.6	17.0	14
Leu	57.0	57.0	55.4	52
Tyr	23.6	23.4	19.5	24
Phe	29.7	27.0	27.6	27
Lys	42.0	44.6	47.9	49
His	17.1	23.1	27.3	17
Arg	22.7	19.4	20.5	23

TABLE II

RESULTS OF CALCULATION OF "SIMILARITY INDEX" BETWEEN THE AMINO ACID COMPOSITION OF EXTRACTED PROTEIN AND 30 REPORTED COMPOSITIONS OF SERUM PROTEINS

Only the serum proteins that showed the highest and next to highest "similarity indices" are listed.

Protein extracted	*Similarity index between amino acid compositions*	*Serum protein*
From spot 80	0.970	Transferrin
	0.829	α_1-Glycoprotein easily precipitated
From spot 81	0.960	Transferrin
	0.855	α_1-Glycoprotein easily precipitated
From spot 82	0.910	Transferrin
	0.848	Haemopexin

to avoid complexity. As shown in Table II, each protein extracted from spots 80–82 showed the highest similarity index with transferrin. Among the three spots, spot 80 showed the highest similarity index with transferrin and the results are in accordance with the fact that the largest amount of protein was extracted from spot 80. We used these results to identify serum proteins on the two-dimensional electrophoretic gels. A similarity index exceeding 0.95 was our standard of identification.

DISCUSSION

In preliminary studies, we examined various procedures for the extraction of protein from the gel matrix and found that every solution and item of glassware used in the extraction steps contained contaminants. Even ion-exchanged and doubly distilled water contained contaminants when it had been stored in a reservoir. The stored water did not show detectable free amino acids but when hydrolysed, amino acids at concentrations up to 3 μg/ml were detected. These results suggest that the contaminants in water are macromolecular substances, such as spores of moulds. Therefore, fresh water taken directly from the condenser of the distillation apparatus was used to prepare extraction solutions. Test-tubes were also sources of contamination. Methods for washing test-tubes with detergents or concentrated hydrochloric acid were examined. However, some contaminants remained in the washed test-tubes in amounts that were not reproducible, possibly because these methods needed water in the last step of washing. Therefore, we calcined the test-tubes without washing them. This was an effective method of removing contaminants.

As shown in Fig. 2, the background gel extract contained about 0.4 μg of contaminants in the first cycle of extraction; the amount was reduced by repeating the cycle, and it became constant at about 0.1 μg. A blank test-tube subjected to the procedures of extraction and hydrolysis in the absence of a piece of gel also contained about 0.1 μg of contaminants. Possibly the contaminants come from air that enters

the test-tubes during the extraction steps. In fact, the amount could be reduced when smaller (55 × 4 mm I.D.) test-tubes were used.

The amino acid compositions of the contaminants found in stored water, in washed test-tubes and in background gel extracts were similar, with high levels of serine and glycine (for example, see Fig. 5B). Results of the amino acid analyses of the contaminated substances will be presented elsewhere.

We used 0.1 *M* sodium hydroxide solution for extraction of proteins. The concentration of sodium hydroxide in the extraction solution was chosen to neutralize a piece of gel that had been soaked in 7% (v/v) acetic acid in the first cycle. Protein was not extracted in the first cycle, so this step served to wash the piece of gel. In the second cycle, the pH of the gel piece increased to 13 and protein was extracted as shown in Fig. 2. Therefore, the second cycle was the actual extraction step. The reason why protein was not extracted in the first cycle is unclear. Possibly fixed and stained proteins could not be solubilized by just neutralizing the pH of the gel, because the rate of the conformation change of the proteins at neutral pH was low.

As shown in Tables I and II, the similarity indices were useful for comparing the amino acid compositions. The values listed in Table II were calculated by comparing 15 amino acids. However, as shown in Fig. 5, the degree of background contribution differs for each amino acid; the measured value of valine is more reliable than that of glycine. Therefore, for the calculation of similarity indices, the reliability of the measured values of each amino acid must be taken into account, especially when the amount of the extracted protein is small.

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