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Standardization of cation-exchange clean-up prior to gas chromatography of amino acids

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ABSTRACT

The optimum conditions for the cation-exchange clean-up of amino acids, present in protein hydrolysates, prior to their gas chromatographic determination were investigated. The results of exchaustive study monitoring the amounts of amino acids as N,O(S)-trifluoroacetyl isobutyl esters, revealed that the recovery of amino acids from the column was affected appreciably by either the particle size or the divinylbenzene content of the resins. Quantitative recovery and reproducible determination of amino acids require (i) a 50-fold excess of resin (calculated as equivalent capacity relative to the amino acids present) and (ii) sufficient amounts of eluates: the volumes both of distilled water and of 7 *M* ammonia solution must be about six times the volume of the wet resin applied. Under optimum conditions the recoveries of alanine, glycine, threonine, serine, valine, leucine (isoleucine), proline, hydroxyproline, methionine, aspartic acid, phenylalanine, ornithine, glutamic acid, tyrosine, lysine, arginine and cystine, were quantitative, both without and after hydrolysis, and that of tryptophan was *ca.* 85%.

INTRODUCTION

The advantages of the gas chromatographic (GC) analysis of amino acids in protein hydrolysates are well known [1–11]. Both the capital and running costs of GC equipment are considerably lower, elution times are shorter and the precision and reproducibility are at least the same, or better, in comparison with the classical ion-exchange technique using an automatic amino acid analyser.

From our previous studies [11–14] of the requirements for the GC analysis of protein hydrolysates, obtained from various matrices, we realized that there is a need to reinvestigate some contradictory statements [1,4,7,8] regarding the effect of an ion-exchange "clean-up" step on the recovery of amino acids. The particular aspects of the necessary cation-exchange procedure [8], especially with regard to the recovery of tryptophan, arginine and cystine [4,7], that require consideration are as follows: (i) the ratio of the amount of sample to that of the resin (*i.e.*, amino acid equivalent/resin capacity equivalent), (ii) elution rate, (iii) eluent volume and (iv) resin type, column dimensions and resin bed size.

The aim of this work was to undertake an exhaustive study to optimize the cation-exchange clean-up of protein hydrolysates with respect to the above parameters.

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EXPERIMENTAL

Materials and reagents

All chemicals used were of analytical-reagent grade from Reanal (Budapest, Hungary), Serva (Heidelberg, Germany), Merck (Darmstadt, Germany), Sigma (St. Louis, MO, U.S.A.) and Applied Science Labs. (State College, PA, U.S.A.). Protein matrices were purchased from commercial sources.

Apparatus

A Chromatron (Berlin, Germany) G.C.H.F. 18.3 gas chromatograph equipped with a flame ionization detector and a 2 m × 4 mm I.D. stainless-steel column was used. Nitrogen was the carrier gas at a flow-rate of 60 cm^3/min. The column packing was 3% SE-30 on Chromosorb W (100–120 mesh) (Supelco, Bellefonte, PA, U.S.A.). The column temperature was increased from 105 to 250°C at 6°C/min. The temperatures of the injector and detector were 270 and 270°C, respectively.

Hydrolysis

A 250-μl volume of a stock solution of amino acids (each 500–1000 μg in 250 μl) in 1 *M* hydrochloric acid was evaporated to dryness and 5 cm^3 of distilled hydrochloric acid were added to the residue. The solution of amino acids (10–12 mg in total) was made oxygen free by bubbling nitrogen (99.99%) through for 5 min. The screw-capped bottles were then closed immediately and placed in an oven at 145 ± 0.5°C for 4 h. Thereafter the hydrolysates were filtered (if necessary) into 25-cm^3 vessels, which could be fitted with either a vacuum distillation device or a reflux condenser, via a ground-glass joint. For filtration glass-fibre paper (grade GF/A; Whatman, Maidstone, U.K.) was used and the insoluble residues were washed with 3 × 1 cm^3 of 0.1 *M* hydrochloric acid. The supernatants were evaporated to dryness for ion-exchange clean-up.

Cation-exchange clean-up

The evaporated residues were dissolved in 1 cm^3 of 0.1 *M* hydrochloric acid and transferred quantitatively with 4 × 1 cm^3 of 0.1 *M* hydrochloric acid onto a cation-exchange resin bed equivalent to $6 \cdot 10^{-3}$ *M* capacity, representing a 50-fold excess of amino acids ($1.2 \cdot 10^{-4}$ *M* in total, calculated with an average molecular weight of 100, *i.e.*, $1.2 \cdot 10^{-2}/100 = 1.20 \cdot 10^{-4}$ *M*). The quality [Amberlite CG-120(H^+), 100–120 mesh; Dowex 50-X8, 30–60 mesh; Varion KS X2, X8, X10, 35–60 mesh] and the particle size (Varion KS X8 35–60, 25–35 and 18–25 mesh) of the resins were varied. In all instances glass columns of 15 cm × 0.75 cm I.D. were used, equipped on the bottom with a glass capillary valve appropriate for the exact adjustment of the flow-rate of the eluent.

The amino acid-containing solution was passed through the resin bed at a rate of 1 cm^3/min, followed 2 min later by 50 cm^3 of distilled water at a rate of 3 cm^3/min.

The amino acids were eluted from the column with 60 cm^3 of 7 *M* ammonia solution at a flow-rate of 1 cm^3/min. The eluate was collected in a 250-ml round-bottomed flask and evaporated to about 1 cm^3. Thereafter the concentrated solution of amino acids was transferred quantitatively with 4 × 1 cm^3 of 0.3 *M* ammonia solution into a 25-cm^3 round-bottomed vessel and evaporated to dryness for derivatization.

Derivatization

To the evaporated residues in the reaction flask, 10 cm^3 of isobutanol [containing 10% (w/w) hydrochloric acid] were added. A reflux condenser was then fitted to the reaction flask and the apparatus was placed in an oil-bath. Esterification took place at 110°C for 60 min. After cooling to room temperature, the solution was evaporated to a syrupy consistency under vacuum in a water-bath at 60°C. To the residue 500 μl of dichloromethane and 1.0 cm^3 of trifluoroacetic anhydride (TFAA) were added and, after sealing the reaction flask acylation was carried out for 10 min at 150°C. Thereafter, the solution of the acylated derivatives was transferred quantitatively into a glass-stoppered, calibrated test-tube (also connected to the vacuum evaporator). The solution of the N,O(S)-TFA esters was evaporated to 500 μl in an ice-bath.

To the residues of the samples dichloromethane and TFAA (in a volume ratio of 4:1) were added, and a stock solution of 0.75 cm^3 was prepared from each. Aliquots of 5–10 μl were injected into the gas chromatograph.

RESULTS AND DISCUSSION

The first step in the model tests was to define the necessary amount and the optimum flow-rate of eluates to obtain maximum (quantitative) amounts of amino acids (Table I).

The types of resin chosen [Table I: Amberlite CG-120(H^+), 100–120 mesh; Dowex 50-X8, 35–60 mesh; Varion KS X8, 35–60 mesh] were based partly on literature data [1–3,6,7] and partly on our preliminary study; all three resins were used after measuring their capacity, ensuring they were present in a 50-fold equivalent excess relative to the *ca.* 12 mg of amino acids present in total. As a result of the measurements the amounts of wetted Amberlite, Dowex and Varion resins were 6, 9 and 9 cm^3, respectively.

The data obtained revealed that the necessary amount of water (fraction I, needed in order to eliminate interfering constituents, such as salts and sugars) contained negligible amounts of amino acids, in particular using the Varion KS resin (Table I, fraction I). With regard to the essential amount of 7 *M* ammonia solution required, for all three resins it proved to be 60 cm^3 (Table I, fractions II and III). The amounts of amino acids found in the third 3-cm^3 7 *M* ammonia solution (fraction IV) using a flow-rate of 1 cm^3/min were less than the experimental error of the measurements (Table II, relative standard deviations).

The effect of the particle size of the resins on the recovery of amino acids was investigated with Varion KS X8 resins of particle sizes of 35–60, 25–35 and 18–25 mesh (Table II, values in columns A, B and C). As can be seen, no differences as a function of resin particle size were observed.

Regarding the influence of divinylbenzene (DVB) percentages, *i.e.*, the amount of cross-linking in the resin, indicated by X2 (2% DVB), X8 (8% DVB) and X10 (10% DVB) in the resin name, the results obtained were as follows. With the X2 and X8 resins no uptake was observed, except for tryptophan (Table II, columns D and E). The recoveries of all the amino acids tested were quantitative, within the experimental error of the measurements (including cation exchange, derivatization and GC steps). The loss of tryptophan was on average *ca.* 15%.

TABLE I

DISTRIBUTION OF AMINO ACID CONTENT OF MODEL SOLUTIONS MEASURED AS N,O(S)-TFAIBE (DERIVATIVES) IN CONSECUTIVE ELUATE FRACTIONS (I–IV) OBTAINED FROM DIFFERENT CATION-EXCHANGE RESINS

Fractions: (I) 50 cm^3 water; (II–IV) 3 × 30 cm^3 of 7 *M* ammonia solution. Values are means obtained from at least three parallel ion-exchange tests: the differences were smaller than 3.0% [relative standard deviation (R.S.D.)]. Blank values, less than 0.01%. Values in parentheses obtained with an elution rate of 7 *M* ammonia solution of 3 cm^3/min.

Amino acid	Amino acid [% (w/w), expressed as a percentage of the amount applied]											
	Amberlite CG-120(H^+) (100–200 mesh)				Dowex 50-X8 (35–60 mesh)				Varion KS X8 (35–60 mesh)			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
Alanine	–	97.6	2.4	–	0.02	99.3	0.60	0.10	–	99.7	0.20	0.06
Glycine	–	98.2	1.8	–	–	99.6	0.30	0.05	–	99.9	0.07	–
Threonine	0.03	97.0	3.0	–	0.05	98.9	0.80	0.20	–	99.9	0.13	–
Serine	0.06	97.1	2.8	0.08	0.03	99.2	0.60	0.20	–	99.9	0.12	–
Valine	0.02	96.7	3.2	0.06	0.01	98.8	0.90	0.30	–	99.8	0.16	–
Leucine + isoleucine	0.05	96.5	3.2	0.20	0.02	98.0	1.6	0.40	0.01	99.7	0.20	0.08 (1.5)
Proline	0.20	97.4	2.3	0.07	0.10	98.4	1.1	0.40	–	99.9	0.12	–
Hydroxyproline	0.10	97.1	2.7	0.13	0.03	98.5	1.0	0.50	–	99.9	0.13	– (2.0)
Methionine	–	96.8	3.2	–	0.02	97.8	2.0	0.20	0.01	99.7	0.30	–
Aspartic acid	0.08	96.8	3.0	0.11	0.10	97.0	2.5	0.40	–	99.5	0.43	0.06 (0.9)
Phenylalanine	0.14	97.3	2.5	0.08	0.14	96.2	3.1	0.60	0.01	97.8	2.0	0.2 (1.5)
Ornithine	–	95.4	4.6	–	–	100.0	–	–	–	100.0	–	–
Glutamic acid	0.10	95.8	4.0	0.09	0.10	96.2	3.2	0.50	0.02	99.1	0.70	0.19 (0.8)
Tyrosine	0.08	96.9	2.9	0.08	0.07	98.1	1.2	0.60	–	98.5	1.3	0.20
Lysine	0.05	96.7	3.2	–	–	99.5	0.50	–	–	99.9	0.06	–
Arginine	–	95.9	4.0	0.11	–	98.8	1.1	0.05	–	94.5	5.0	0.45 (1.6)
Tryptophan[a]	–	95.3	3.7	1.0	0.09	96.8	3.0	0.10	–	87.7	11.8	0.50 (2.0)
Cystine	–	90.7	9.0	0.30	–	99.2	0.70	0.05	–	96.0	3.5	0.50

[a] Totals, relative to the value without ion exchange, from Amberlite, Dowex and Varion resins, were 75, 85 and 85%, respectively.

With the 10% DVB-containing resin (Table II, column E) considerable shortages, but much lower than reported previously [1,2,4,6,7], were found with respect to the recovery of tryptophan, arginine and cystine (Table II, column E, recoveries in quotation marks).

Comparing the recovery data after hydrolysis (Table II, values A after hydrolysis) with those obtained without hydrolysis, it is obvious that with the exception of tryptophan no additional losses need to be taken into consideration. The recovery of tryptophan after hydrolysis performed in the presence of 3-(2-aminoethyl)indole, followed by cation-exchange clean-up, is currently being studied.

Exhaustive studies have been made to verify the earlier literature data [5,7] concerning the impact of ammonia on the amount of arginine during the evaporation

TABLE II

RECOVERY OF AMINO ACIDS AFTER CATION-EXCHANGE CLEAN-UP APPLYING VARIOUS KINDS OF VARION KS RESINS WITHOUT (A–E) AND AFTER (A) HYDROLYSIS

The main values were obtained from at least three parallel ion-exchange tests, for conditions A–E, before and after hydrolysis, equally. A–E: clean-up test using different kinds of Varion KS resin: A, B and C, Varion KS X8 with particle sizes (A) 35–60 mesh, (B) 25–35 mesh and (C) 18–25 mesh; D and E, Varion KS X2 and Varion KS X10 resin, respectively, with particle size 35–60 mesh. Values in parentheses are percentage recoveries of tryptophan, relative to the value obtained without cation exchange. Values in quotation marks are percentage recoveries of arginine, tryptophan and cystine expressed as percentages of the mean values.

Amino acid	Weighed (μg)	Obtained after elution (μg)								
		Without hydrolysis					After hydrolysis, A	Mean	S.D.	R.S.D. (%)
		A	B	C	D	E				
Alanine	541.9	539	554	546	546	544	550	546	4.4	0.80
Glycine	506.5	506	502	501	506	508	516	508	5.6	1.1
Threonine	511.8	494	516	514	510	523	502	511	9.0	1.7
Serine	515.0	515	515	517	501	511	517	513	5.4	1.1
Valine	539.7	539	539	541	525	535	542	538	5.6	1.0
Leucine + isoleucine	945.9	954	956	953	920	946	946	948	12.9	1.4
Proline	582.4	598	578	588	590	581	593	587	6.6	1.1
Hydroxyproline	534.4	542	549	536	533	539	526	537	6.6	1.2
Methionine	426.7	433	437	428	420	427	426	428	5.0	1.2
Aspartic acid	884.1	880	893	887	877	879	865	883	10.4	1.2
Phenylalanine	819.6	810	828	836	831	810	809	824	12.8	1.6
Ornithine	558.8	569	559	565	559	565	572	564	4.9	0.86
Glutamic acid	771.0	772	762	790	781	770	775	777	9.9	1.3
Tyrosine	527.1	541	528	538	537	532	523	534	5.9	1.1
Lysine	756.0	764	768	801	800	802	746	777	22.0	2.8
Arginine	606.5	597	596	593	590	568[a] "94.8"	604	599	7.9	1.3
Tryptophan	543.7	477 (86.6)	474 (86.0)	477 (86.6)	477 (86.6)	462[a] (83.8) "97.6"	–	476 (85.8)	1.3	0.27
Histidine	791.3	803	798	798	778	760	772	787	15.6	2.0
Cystine	771.2	783	751	756	733	686[a] "90.1"	759	761	17.9	2.3

[a] Left out of the mean.

process: no losses could be found using 1–7 *M* ammonia, in agreement with the present results, with a quantitative recovery of arginine obtained also after the cation-exchange clean-up procedure (Table II, conditions A–D).

CONCLUSION

The surprisingly high losses obtained with Dowex 50-X2 resin [4] for tryptophan (30–35%), cysteine and arginine (30–50%) was decreased for tryptophan (to 30%) and for arginine (to 10%) by applying pressure during elution [7]. The large losses, in general, can be explained by the statement, published in a critical review [8], that "less than half of the publications carried any account of recovery or analytical precision".

The main reason for losses, in our experience, is that the requirement for either a sufficient amount of resin (in the knowledge of its equivalent capacity and the excess of resin needed) or a satisfactory amount of eluate have not been fulfilled.

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