Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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To cite this Article Muzzarelli, Riccardo A. A. , Tanfani, Fabio , Muzzarelli, Maria G. , Scarpini, Gianfranco and Rocchetti, Roberto(1978) 'Ligand-Exchange Chromatography of Amino Acids on Copper-Loaded Chitosan', Separation Science and Technology, 13: 10, 869 — 879
To link to this Article: DOI: 10.1080/01496397808057135
URL: http://dx.doi.org/10.1080/01496397808057135

SEPARATION SCIENCE AND TECHNOLOGY, 13(10), pp. 869–879, 1978

Ligand-Exchange Chromatography of Amino Acids on Copper-Loaded Chitosan

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Abstract

Amino acids are retained on the copper form and on the amino copper form of chitosan, especially aspartic acid, glutamic acid, tryptophan, and cysteine. The best conditions for collection and for elution are in phosphate buffers at pH 7 and 12, respectively. No leakage of copper occurs; the amino acids are recovered as copper complexes with a ratio of 1: 2 copper: amino acid. Several advantages of chitosan over the resin Chelex are pointed out; namely, absence of swelling, great copper capacity. hydrophilicity, and porous structure.

INTRODUCTION

Since the discovery of the chelating ability of substituted celluloses (*1*, *2*) and early work on ligand-exchange chromatography (*3*), the possibility has existed of taking advantage of the combined chelating power and hydrophilic loose structure of certain natural polysaccharides for the separation of amino acids, peptides, and proteins. This has been recognized by many authors, and numerous attempts at the modification of agarose, sepharose, and cellulose have been carried out for this purpose (*4–7*). Artificial resin-based chelating polymers, on the contrary, have a high degree of cross-linking and hydrophobic character; thus they are less apt to be useful for the fractionation of large molecules. In spite of this fact,

the high metal ion capacity of the chelating resin Chelex, a styrene–divinylbenzene copolymer, has made certain authors believe that the disadvantages due to the matrix may be compensated for by the metal ion concentration attainable in the resin, which is higher than for the substituted celluloses. Thus a number of articles have recently been published proposing ligand-exchange separations of amino acids on that synthetic resin in the copper or nickel forms (*8–11*).

We have described in the past the high capacity for transition metal ions exhibited by chitosan (*12–17*), and its superior characteristics in comparison to those of Chelex have been emphasized in several occasions (*15, 17–21*) to the point that certain presumed performances of Chelex had to be reassessed (*22*). As far as ligand-exchange chromatography is concerned, chitosan exhibits several characteristics which qualify it as a most attractive chromatographic support (*17*). Some of them are recalled here for clarity: in addition to its outstanding indifference to alkali and alkali-earth ions, chitosan has very high capacity for copper which is strongly held over a wide range of pH values, including the alkaline region. Chitosan does not swell upon interaction with solutes even under sudden and drastic alterations of ionic force and pH; it is also thermally stable. The polysaccharide crystalline networks carrying regularly spaced amino groups form a sponge-like hydrophilic structure and permit water to enter the interstices. The lack of swelling is also evident from quantitative estimates of the low back pressure in columns. Chitosan has the most surface nitrogen available for chelation, as determined by ESCA measurements.

In view of this interesting information on chitosan, we have undertaken the present research to assess its behavior toward some amino acids as a ligand-exchanger in the copper form.

EXPERIMENTAL

Chitosans

Preparation of Copper-Chitosan (Cu-Chitosan)

Chitosan powder (10 g) was stirred for 1 hr with a solution of copper sulfate pentahydrate (7.5 g in $\simeq$200 ml). The resulting deep blue copper-loaded polymer was washed several times with distilled water until no copper was detected by hot graphite atomic absorption.

Preparation of Amino Copper-Chitosan (AM-Cu-Chitosan)

The tetraamino copper complex was prepared by dissolving copper sulfate pentahydrate (7.5 g) in distilled water ($\simeq$200 ml) and adding

excess ammonia. Chitosan powder (10 g) was then stirred in this solution and the resulting violet amino copper-chitosan was then washed as above. As an alternative, the copper-chitosan was stirred in the tetraamino copper solution with identical result.

Conditioning of Chitosans

Before being packed in a column, the chitosans were adjusted to the desired pH by stirring with either sulfuric acid, sodium hydroxide, or phosphate buffers.

Solutions

Buffers were prepared with potassium dihydrogen phosphate (0.05 *M*) and disodium hydrogen phosphate (0.05 *M*) to obtain the desired pH values. The amino acids were 0.4 m*M* for batch measurements, 0.2 m*M* in phosphate buffer for batch measurements with phosphate-conditioned chitosan at pH 7.0, 0.3 m*M* in water for breakthrough curves (4 ml), and 1.0 m*M* in either phosphate buffer or in water at the desired pH for chromatographic experiments (3 ml).

Instrumentation

Chromatographic experiments were performed with the aid of Gilson Minipuls 4 pumps and columns, 16 × 0.6 cm or 9 × 0.4 cm, at flow rates of 0.6, 1.0, or 1.5 ml/min. The amino acids determinations were based on the ninhydrin reaction and performed with the aid of a Beckman 25 spectrophotometer and a Baush & Lomb Spectronic 20 at 540 nm. Copper determinations were performed with a Perkin-Elmer 305 atomic absorption spectrometer, equipped with deuterium back-ground compensation and hot graphite atomizer.

RESULTS AND DISCUSSION

Batch and Breakthrough Experiments

Preliminary information about the interactions of amino acids with copper-loaded chitosan has been obtained with the batch technique on Cu-chitosan and on AM-Cu-chitosan. Moreover, breakthrough curves were plotted for 16 × 0.6 cm columns ($\simeq$ 0.5 g); all these data are presented in Figs. 1 and 2 and in Table 1. They show that all the amino acids under investigation are more or less collected on these supports.

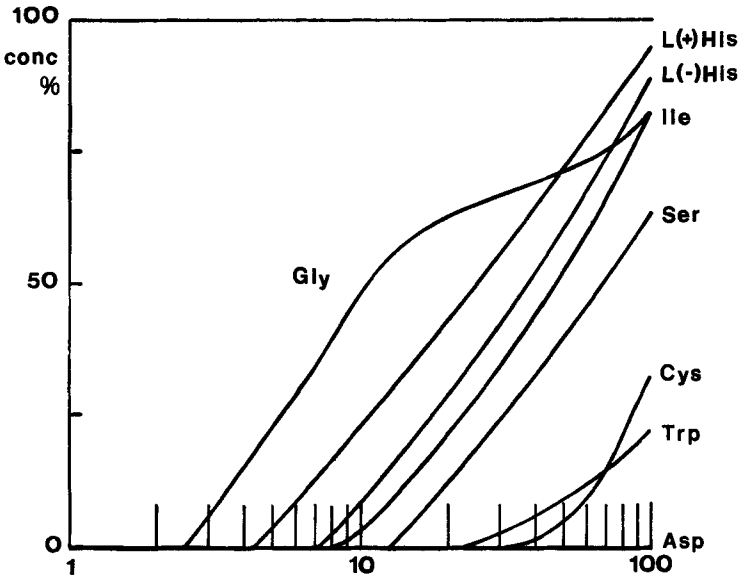

FIG. 1. Breakthrough curves for amino acids (0.3 m*M* in water) on Cu-chitosan columns (16 × 0.6 cm, ~0.5 g, flow rate 0.6 ml/min). The pH of the effluent was 5.8 for all amino acids.

TABLE 1

Collection Percentages of Amino Acids on Cu-Chitosan and on AM-Cu-Chitosan as Obtained by Shaking 100 mg Powder with 1.2 μmoles of Amino Acid (3 ml, 0.4 m*M*) for 1 hr at Room Temperature

Amino acid	Cu-chitosan	AM-Cu-chitosan
L-Histidine	62	59
L(+)-Cysteine	94	10
L(+)-Glutamic acid	100	87
Glycine	10	37
L(+)-Aspartic acid	100	87
L(−)-Tryptophan	100	65
L(+)-Isoleucine	69	19
DL-Serine	88	59

Glutamic acid, aspartic acid, tryptophan, and cysteine are very efficiently collected. The breakthrough curves relevant to Cu-chitosan in Fig. 1 form two sets, the first of which refers to the said amino acids for which the Cu-chitosan capacity is high. Even higher capacities are shown in Fig. 2 for all the amino acids tested on AM-Cu-chitosan.

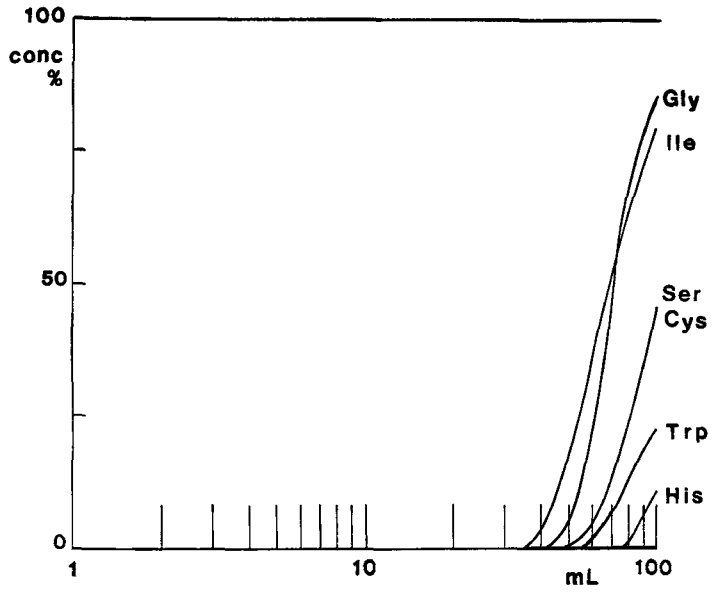

FIG. 2. Breakthrough curves for amino acids (0.3 mM in water) on amino Cu-chitosan columns (16 × 0.6 cm, ~0.5 g, flow rate 0.6 ml/min). The pH of the effluent was 7.8 for all amino acids.

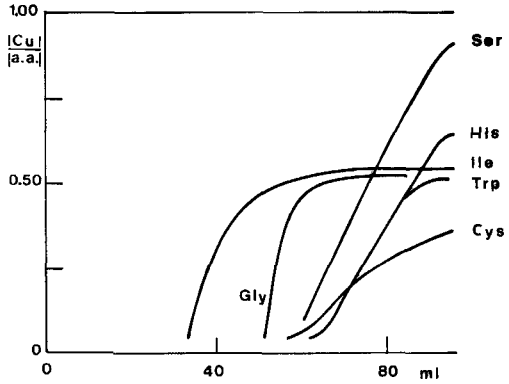

FIG. 3. Molar ratios of copper to amino acids in the elution fractions for the Cu-chitosan columns.

The determinations of copper in the fractions from the breakthrough experiments show that copper is very low and the copper/amino acid ratio tends to be 0.5, as indicated in Fig. 3. All the copper determinations

indicated that no leakage of copper occurs under the adopted conditions and that the eluted amino acids are accompanied by a minor amount of copper due to complexation.

Selection of Optimum pH Values for Chromatography

Small columns, 9 × 0.4 cm (≃ 0.2 g), were used to extend the preliminary data to a chromatographic process. They were made of Cu-chitosan conditioned with buffer solutions at pH 5.0, 6.0, 7.0, and 8.0. The amino acids solutions (12 ml) at the same pH as the column were percolated at 1.0 ml/min and collected in four fractions of 3 ml each. Buffer solution was then passed to collect four more fractions. Elution was performed with eight 3-ml fractions of 0.05 *M* disodium hydrogen phosphate at pH 12.0. Both the amino acid and copper were determined on these fractions. The data are in Table 2, and some of them are illustrated in Figs. 4 and 5. Table 2 shows that under these conditions the amino acids are retained in

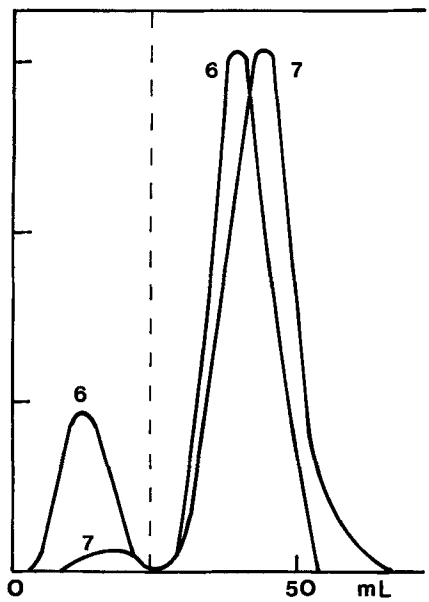

FIG. 4. Chromatographic profiles of cysteine on Cu-chitosan columns buffered at pH 6 and 7 (9 × 0.4 cm, flow rate 1 ml/min). Elution performed with 0.05 *M* disodium hydrogen phosphate at pH 12.0. Retention percentages are in Table 2.

TABLE 2

Retention Percentages of Amino Acids on Cu-Chitosan and on AM-Cu-Chitosan as Obtained by Percolating 1.0 m*M* Solution (12 ml) through a 9 × 0.4 cm Column and Washing with 12 ml Buffer. The Data for Cysteine and Aspartic Acid Correspond to Those in Fig. 7

	Cu-chitosan, buffered with phosphate 0.05 *M* at pH				AM-Cu-chitosan, unbuffered			
Amino acid	5.0	6.0	7.0	8.0	6.0 6.7	7.0 8.5	9.1 9.5	10.1 10.6
His	20	46	44	41	34	43	39	36
Cys	—	74	95←	90	87	44	43	43
Glu	33	56	46	49	96	31	9	11
Gly	38	22	28	43	34	17	22	4
Asp	76	89	80←	63	23	31	5	4
Trp	32	57	55	66	66	59	33	4
Ile	—	—	—	—	36	33	22	5
Ser	8	22	42	49	52	36	21	6

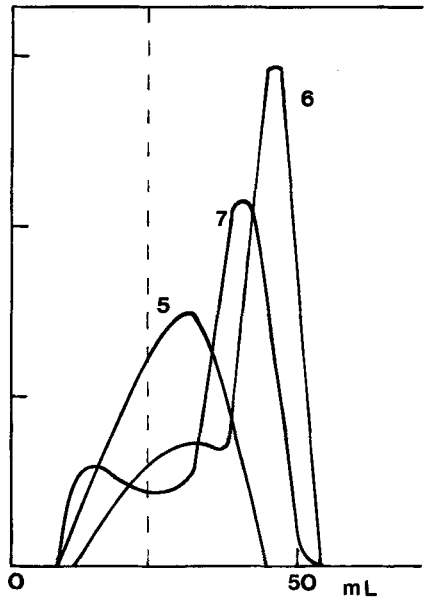

FIG. 5. Chromatographic profiles of aspartic acid on Cu-chitosan columns buffered at pH 5.0, 6.0, and 7.0 (9 × 0.4 cm, flow rate 1 ml/min). Elution performed with 0.05 *M* disodium hydrogen phosphate at pH 12.0. Retention percentages are in Table 2.

the column to an appreciable extent in the pH interval 5 to 8; the optimum pH for fixation lies between 6 and 7 except for glycine which seems to be the less-fixed amino acid in this group.

The collected amino acids can be eluted with good yields: the curves in Figs. 4 and 5 are in part adsorption curves (at pH 6 and 7 for cysteine and at pH 5, 6, and 7 for aspartic acid) and in part elution curves at pH 12. Thus a proper variation of pH by disodium hydrogen phosphate buffer permits recovery of the amino acids. The elution percentages are calculated with respect to the amount of amino acids actually present in the column.

Based on the breakthrough curves shown in Figs. 1 and 2, one can expect higher capacities for AM-Cu-chitosan and delayed elution curves. This was found to hold true, and Fig. 6 shows the shift of the chromatographic curve of glycine, which is merely delayed on Cu-chitosan but which requires elution on AM-Cu-chitosan. However, if the data in Table 2 are compared, it appears that amino acids interact more strongly with Cu-chitosan than with AM-Cu-chitosan, even if the capacity of the latter is higher.

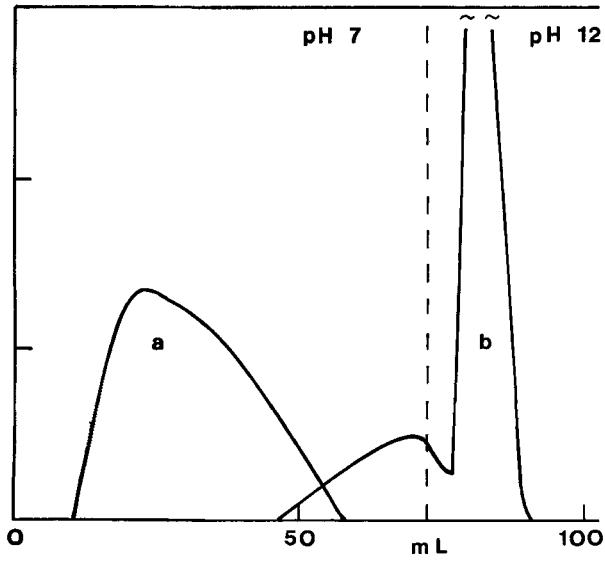

FIG. 6. Chromatographic curves of glycine on (a) buffered Cu-chitosan and (b) AM-Cu-chitosan columns (16 × 0.6 cm, flow rate 1.5 ml/min, room temperature).

The chromatographic sequence used on 16 × 0.6 cm columns was as follows: adsorption of a single amino acid from 1.0 ml of its 6 m*M* solution, washing with phosphate buffer at pH 7.0 (72 ml), and elution with phosphate at pH 12.0 (72 ml). Figures 6 and 7 show some of the curves obtained (superimposed) which describe the behavior of the amino acids.

As far as a comparison with Cu-Chelex is concerned, the papers cited (*10*, *11*) do not provide information about the leakage of copper and nickel from the resin Chelex and about elution profiles: the elution is dealt with in part of one line where it is said that it was performed "with 50 ml of 3 or 6 *M* ammonia." Those two papers (*10*, *11*) report a total of five chromatograms which are not relevant to the proposed ligand-exchange process, but are the chromatograms recorded with an amino acid autoanalyzer on the bulk eluate for analytical purposes only and have nothing to share with the ligand-exchange process.

We have verified in the laboratory that copper is washed off AM-Cu-Chelex during elution to such an extent that the eluate is a blue solution. Due to swelling, the column length varies by 30 to 40% and clogging occurs.

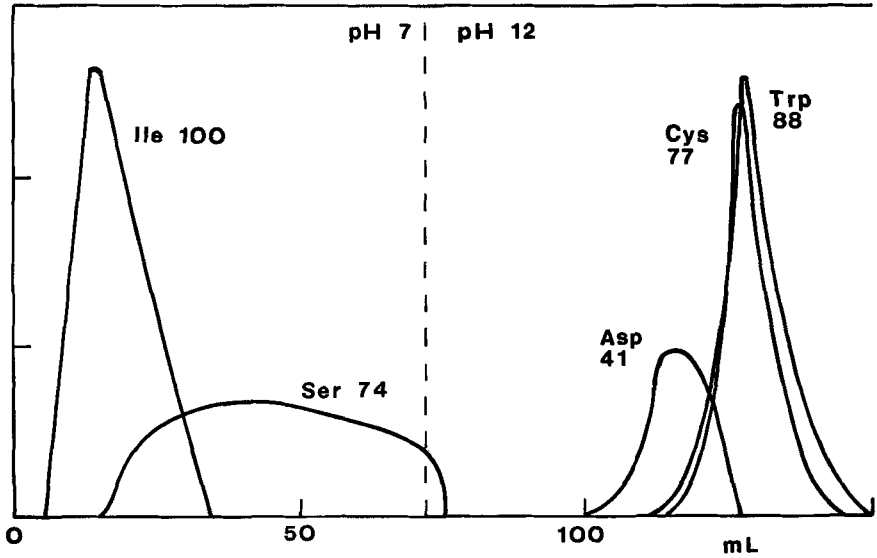

FIG. 7. Superimposed chromatographic curves for several amino acids on buffered Cu-chitosan columns (16 × 0.6 cm, flow rate 1.5 ml/min, room temperature).

Therefore, both the copper-loaded chitosans, either in the hydroxyl or ammonia form, are superior to the AM-Cu-Chelex resin because they retain copper under all the conditions tested, do not swell, and thus constant flow-rates are obtained. Moreover, they possess selectivity for certain amino acids and enable one to perform separations, which was not demonstrated for the AM-Cu-Chelex resin.

Another point of difference is the behavior toward aspartic and glutamic acids. While these acids have been reported not to be collected on AM-Cu-Chelex, they are on Cu-chitosan and on AM-Cu-chitosan. This information is interesting in relation to separations of amino acids from each other and for the recovery of proteins. The present results are certainly encouraging for planning more extended research on the uses of chitosan for the immobilization and separation of peptides.

CONCLUSIONS

Both Cu-chitosan and AM-Cu-chitosan are ligand exchangers; these new supports can collect certain amino acids with good yields. They differ in that Cu-chitosan shows a more marked selectivity while AM-Cu-chitosan shows an overall greater capacity. During the collection of the amino acids and as a consequence of the pH variations introduced by the buffers selected, there is no leakage of copper or ammonia and there is no observable swelling or clogging. These supports look very attractive for the recovery of certain amino acids and for their separation.

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Received by editor March 20, 1978