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Adsorption of Phenylalanine from Casein Hydrolysates

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ABSTRACT

The specific diet therapy for phenylalaninemies requires special hydrolysates of proteins where phenylalanine content is reduced to approx 0.53% of the amino acids present. In previous work, Amberlite XAD-4 resin was used to retain phenylalanine from an acid hydrolysate of casein. It was also observed that the adsorption isotherm of phenylalanine on the resin showed a convex pattern that allowed a frontal chromatography. In the present study, this technique was improved, aiming at the processing of larger hydrolysates volumes. This was achieved with the use of two sequential columns (50×1 cm), each containing 34 cm^3 of the resin, and joined through a 1-cm long tygon, tube 1 mm diameter. This system was used to process 100 mL of casein acid hydrolysate containing 12 g of free amino acid and allowed the reduction of phenylalanine content from 4.39 to 0.14% of the total amino acids present, within 1 h. It was also observed that this technique could not be directly applied to enzymatic hydrolysates of casein unless they were especially produced for this purpose, which means that in this kind of hydrolysate, phenylalanine should be free or linked in small adsorptive peptides.

Index Entries: Phenylalanine adsorption; low phenylalanine hydrolysate; enzymatic hydrolysate of casein; protein hydrolysates; adsorption.

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INTRODUCTION

In a previous study, it was shown that the adsorption isotherm for phenylalanine (Phe) on Amberlite XAD-4 resin was a convex one (*1*). This fact allowed a frontal chromatography (*2*). At this laboratory, this resin was used to retain Phe from an acid hydrolysate of casein (*1*). In that study, it was shown that Phe content could be reduced from 3.42 to 1.35% of the total amino acids present in this hydrolysate (*1*). This percentage was still high for a flour that is used in specific diet therapy for phenylalaninemies (*3*), but the technique showed promising results.

In the present study, this technique was improved so that larger volumes of the acid hydrolysate could be used. It has also been extended to enzymatic hydrolysate that is assumed to promote a rapid absorption of amino acids (*4,5*) and a less hypertonic diet (*6*).

MATERIAL AND METHODS

Amberlite XAD-4 was obtained from Rohm & Haas Co. (Brazil). Total acid hydrolysates of casein were kindly supplied by Universidade Federal Fluminense (UFF, Brazil) and the pepsin-pancreatin hydrolysate by Biobrás (Montes Claros, MG, Brazil). Two different batches of the total acid hydrolysates were used in this study (hydrolysate A and B). The amino acid composition of hydrolysate A has been previously described (*1*). Table 1 shows the composition of the other acid hydrolysate (hydrolysate B) and of the one enzymatic hydrolysate (hydrolysate C).

Except for the expected absence of tryptophan (Trp) in the acid hydrolysates, all of them fulfill the demands prescribed by Pharmaceutical Code (*7*). The preparation and regeneration of Amberlite XAD-4 have been described previously (*1*). Experiments designed to determine the adsorption isotherm were carried out as described previously (*1*). To perform ultrafiltration of enzymatic hydrolysate, 100 mL of 1% (w/v) solution of enzymatic hydrolysate were submitted to ultrafiltration through a UM2 membrane (AMICON Corporation, Danvers, MA) to retain peptides with mol wt above 1000.

Column Experiments: Operation

For acid hydrolysates, two columns (50×1 cm), each one containing resin packed in a 34 cm^3 vol were connected through a 1-cm long tygon tube, 1 mm diameter. Two different sample vols (30 and 100 mL) were applied to the first one. The downflow rate was 370 mL/h and maintained by means of a peristaltic pump (Harvard Apparatus Co. Inc., Milles, MA). The elution was carried out with distilled water. A vol V (105 and 180 mL) was applied to the second column. This vol V corresponds to eluate

Table 1
Composition of the Acid (B) and Enzymatic (C) Hydrolysate

	Hydrolysate			
	B		C*	
Amino acid	mg/mL	%	mg/mL	%
Lys	10.30	8.67	0.82	9.83
Hys	2.48	2.09	0.19	2.28
Arg	3.92	3.30	0.27	3.24
Asp	11.23	9.46	0.49	5.87
Thr	4.38	3.69	0.30	3.60
Ser	7.02	5.91	0.39	4.67
Glu	32.08	27.03	1.91	22.90
Pro	10.29	8.67	0.81	9.71
Gly	2.12	1.78	0.14	1.68
Ala	4.96	4.18	0.22	2.64
Val	5.22	4.39	0.51	6.11
Met	2.34	1.97	0.22	2.64
Ile	3.92	3.30	0.39	4.68
Leu	9.96	8.39	0.69	8.27
Tyr	3.10	2.61	0.45	5.39
Phe	5.36	4.39	0.43	5.16
Trp	—	—	0.11	1.32

*1% (w/v) solution.

Region A1 of 30 mL or 100 mL application plus two dead vols (36 mL). After its application the two columns were disconnected. The first column was submitted to alkali elution with 90 mL of 0.1*N* NaOH, whereas the second column was eluted with different vols of distilled water. These latter vols correspond to Region A2 in each eluate. Afterwards, the second column was eluted with 90 mL of 0.1*N* NaOH. Six-mL fractions of the aqueous or alkaline eluates were collected in tubes using an Ultrorac Faction Collector (LKB Bromma, Sweden). Figure 1 shows the arrangement of the system.

For enzymatic hydrolysate, 10 mL of a 5% (w/v) solution of the enzymatic hydrolysate were applied to a column (50×1 cm). The downflow rate was the same as for the acid hydrolysates. The elution was carried out with distilled water. Six-mL fractions of this eluate were collected as described for acid hydrolysates.

Analysis of Elution Profile

For acid hydrolysates, the separation profile and the characterization of the different fractions were carried out as described before (*1*). The eluate corresponding to Region A1 (vol V) obtained from different vol

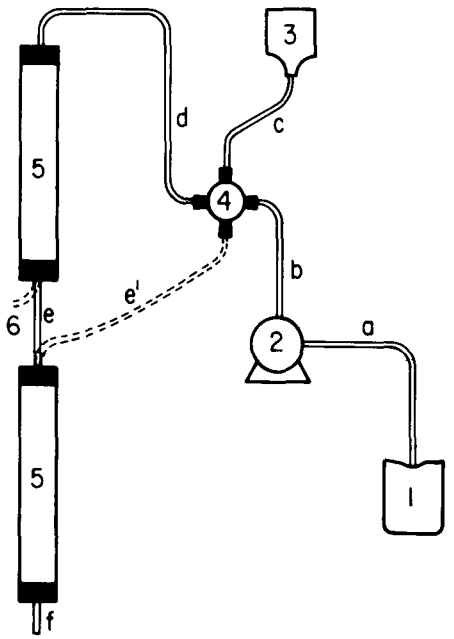

Fig. 1. Schematic diagram of the chromatographic process. (1) eluent vessel; (2) peristaltic pump; (3) sample vessel; (4) four-way valve; (5) glass columns (50×1 cm); (6) tygon tube (1×0.01 cm). Step I-sample application (sequence c, d, e, f); step II-sequential elution of both columns (sequence a, b, d, e, f); step III-elution of each column separately (sequence a, b, e', f).

samples were determined in separate experiments. A constant dilution rate (~1.5X) was obtained. Region A2 vols were calculated according to this dilution rate.

For enzymatic hydrolysate, the separation and characterization of the regions were carried out by measuring UV absorbance at 220 and 260 nm. Three different regions were identified (*see* Fig. 5).

To determine the amino acid composition of fractions, the aqueous or alkaline eluates of the columns were pooled together and treated as described previously (*1*). The eluate obtained from the application of enzymatic hydrolysate was previously submitted to hydrolysis with HCl or methane-sulphonic acid (*8*).

RESULTS AND DISCUSSION

Adsorption of 30-mL Acid Hydrolysate

Region A1 (Fig. 2) contains ~95% of the amino acids originally present in hydrolysate A. The application of 105 mL (vol V—*see Material and Methods*) to the second column allows a better separation of the aromatic

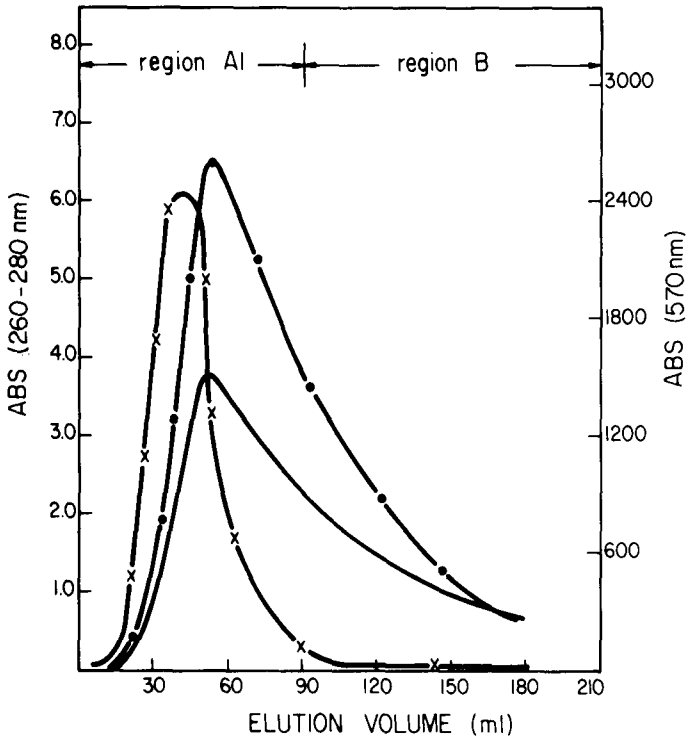

Fig. 2. Elution profile of 30 mL casein acid hydrolysate through one column (50×1 cm) containing Amberlite XAD-4. Flux: 370 mL/h. Elution was carried out with distilled water. The end of Region A1 is indicated by the arrow. Absorbance at 260 nm (—) and at 280 nm (—·—) measured against the eluent. Ninhydrin reaction, absorbance at 570 nm (x-x). All measurements performed at room temperature (24–25°C).

amino acids (Abs at 260 and 280 nm) from the others (Abs at 570 nm) as can be seen by comparing Figs. 2 and 3.

Region A2 (Fig. 3) contains ~89% or 2.0 g of the amino acids originally present in the hydrolysate and almost no Phe. The amino acid analysis of Region A2 and of the alkaline eluate of the first column, which contains the adsorbed amino acids, shows a recovery of ~100% for most of them (Table 2). Met, Ile, Leu (~87% average recovery), Tyr (~70%), and Phe (~60%) are the exceptions. The recovery was taken as 100% for the calculation of the content of each amino acid in the aqueous and alkaline eluates. The composition of the eluates is expressed as the average of the percentages of amino acids grouped according to their chemical nature.

These results confirm that Amberlite XAD-4 is somewhat effective in adsorbing nonpolar amino acids (*9*). On the other hand, the aromatic amino acids show a stronger specific intermolecular interaction with the resin (*10*) (Table 2).

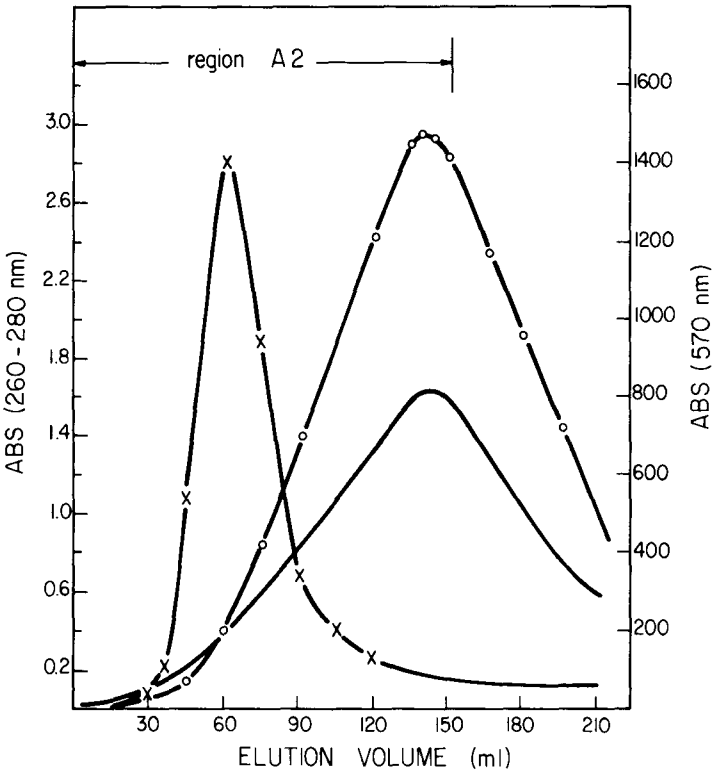

Fig. 3. Elution profile of 30 mL casein acid hydrolysate through two columns (50×1 cm) containing Amberlite XAD-4. For other details *see* Fig. 1.

Table 2
Percentage Distribution
of the Recovered Amino Acid Mass for Region A2
and Alkaline Eluate of the First Column*

Amino acid nature	Eluate Region A2, %	Eluate Alkaline, %
Polar	99.33	0.67
Nonpolar	94.84	5.16
Aromatic		
Phe	0.04	99.96
Tyr	59.69	40.31

*Sample volume: 30 mL.

The adsorption efficiency for Phe of the overall process is ~100%. Previous results showed that the adsorption efficiency for Phe of the first column is ~60% (*1*). Therefore, the adsorption efficiency of the second column is higher and can be estimated as 99.75%. This value was calculated by the equation:

Table 3
Percentage Distribution
of the Recovered Amino Acid Mass for Region A2
and Alkaline Eluates of the First and Second Columns*

Amino acid nature	Eluate Region A2, %	Alkaline, %
Polar	97.11	2.89
Nonpolar	90.13	9.88
Aromatic		
Phe	2.67	97.33
Tyr	60.32	39.68

*Sample volume: 100 mL.

$$\triangle E_t = (\triangle E_1 + \triangle E_2) - (\triangle E_1 \cdot \triangle E_2)$$

This equation is obtained assuming that adsorption efficiency is the ratio between adsorbed mass and applied mass. $\triangle E_t$ is the total adsorption efficiency of the whole system. $\triangle E_1$ and $\triangle E_2$ are the efficiencies of the first and second columns, respectively. The higher efficiency presented by the second column can probably be caused by the lower concentration of aliphatic amino acids and also by the absence of products derived from Trp degradation (*8*) in Region A1.

Thirty-mL application in the system (which correspond to all resin volume of one column) of two sequential columns led to a marked improvement of the methodology with the total removal of Phe from the hydrolysate. This fact allows a greater overloading of the system. The choice of 100-mL application was somewhat arbitrary since various factors can affect adsorption efficiency of the resin (*11*).

Adsorption of 100-mL Acid Hydrolysate

One hundred mL of hydrolysate B were applied to the first column. A vol V of 180 mL was rechromatographed on the second column. Region A2 corresponds to ~89% or ~10 g of total amino acids applied. The amino acid analysis of Region A2 plus the alkaline eluates of the first and second columns shows a recovery of ~100% for most amino acids. The recovery of nonpolar amino acids (Met, Ile, Leu, Tyr, and Phe) is in accordance with the results obtained for 30-mL application (Table 3). The results in Table 3 are presented as described for Table 2.

The adsorption efficiency for Phe of the system is ~97% so the efficiency of the overall system measured as $\triangle E_t$ decreased. However, if we considered the ratio between the total mass of Phe adsorbed and the volume of resin, we can see that it increased from 1.13 mg/cm^3 of the resin (30-mL application) to 7.76 mg/cm^3 of the resin (100-mL application).

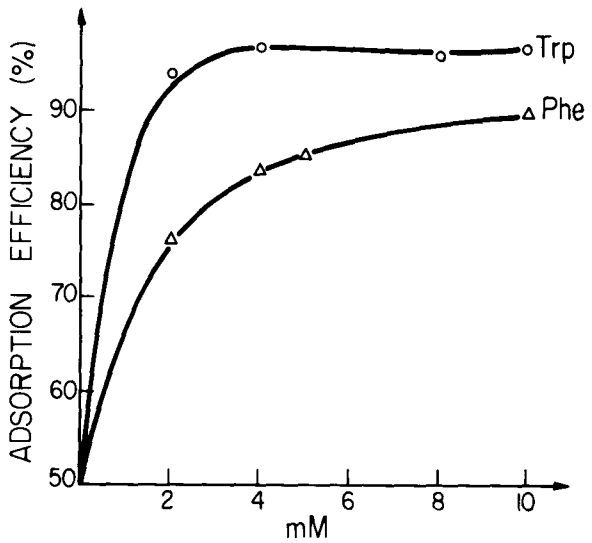

Fig. 4. Adsorption isotherm of Trp on the resin Amberlite XAD-4. Batch experiments. Volume of Trp (○—○) or Phe (△—△) solutions: 2 mL; vol of the resin: 2 cm^3. Room temperature (24–25°C).

Phe content was reduced from 4.39 to 0.14% of the total amino acids. This result is very satisfactory and the methodology has proved to be useful to reduce Phe content to hydrolysates levels that are appropriate to diet therapy of phenylalaninemies. Working on the laboratory scale, the application of ~12 g of sample on 68 cm^3 of resin yields ~10 g of product within 1 h.

Enzymatic Hydrolysate

The nitrogen source of a formula diet is normally provided entirely as free amino acids. Substitution of amino acids by di- or tri-peptides has been pointed out to have the advantages of reduction of hypertonicity (*12*) and enhancement of absorption (*13,14*).

The application of this methodology to enzymatic hydrolysate is therefore indicated. The amino acid composition of this hydrolysate is shown in Table 1 (hydrolysate C). In this hydrolysate tryptophan (Trp) is present, so it will probably affect Phe adsorption by the resin. Therefore, the determination of adsorption isotherm for Trp is recommended.

Determination of Trp Adsorption Isotherm: Batch Experiments

Batch experiments have been carried out in order to determine the adsorption isotherm of the resin for Trp. Its chemical nature allows for a competition with Phe for the adsorption sites of the resin. Figure 4 shows that the adsorption isotherm of Trp is similar to that of Phe. Because of its chemical nature, Trp shows the same or an even higher affinity for the resin.

Table 4
Percentage Composition of Ultrafiltrate and Retentate

Amino acid nature	Fraction	
	Filtrate, %	Retentate, %
Polar	37.65	56.03
Nonpolar	40.35	36.42
Aromatic		
Phe	11.74	3.72
Tyr	7.34	3.82
Trp	2.93	nd

Ultrafiltration of Enzymatic Hydrolysate

Ultrafiltration is in itself a possibility for the separation of peptides according to their mol wt. The use of UM2 membrane allows the separation of peptides with mol wt < 1000 and free amino acids from peptides with a higher mol wt. Ultrafiltration of hydrolysate was carried out in order to obtain a fraction that could be used as sample for the methodology proposed. Table 4 shows the amino acid composition of the resulting ultrafiltrate and retentate fractions. The compositions are expressed as the sum of the percentages of amino acids grouped according to their chemical nature.

The mass of the ultrafiltrate fraction is $\sim 18\%$ of the total mass filtered, and its percentage composition demonstrates that it has been enriched in aromatic amino acids by comparison with the composition of the whole enzymatic hydrolysate. These amino acids are, on average, about three times are concentrated in the ultrafiltrate fraction.

The retentate fraction contains a higher mass content. This mass content is composed mainly of peptides with mol wt above 1000 and a Phe content of 3.72% of the amino acid by mass. Even if Phe content is reduced to a suitable level ($< 0.53\%$) with the use of this methodology, that is, if there is adsorption of Phe there will also be a loss in other amino acids since Phe is not free but bound to other amino acids as a component of oligopeptides. Unfortunately, the low mass content together with its high percentage of Trp (2.93%) turns the ultrafiltrate fraction inadequate as raw material for our purposes. Therefore, the whole enzymatic hydrolysate seemed more appropriate as feedstock for this methodology.

Adsorption of 10-mL Enzymatic Hydrolysate

In order to match the Phe concentration of the enzymatic hydrolysate with its concentration in acid hydrolysates, a 5% (w/v) solution was

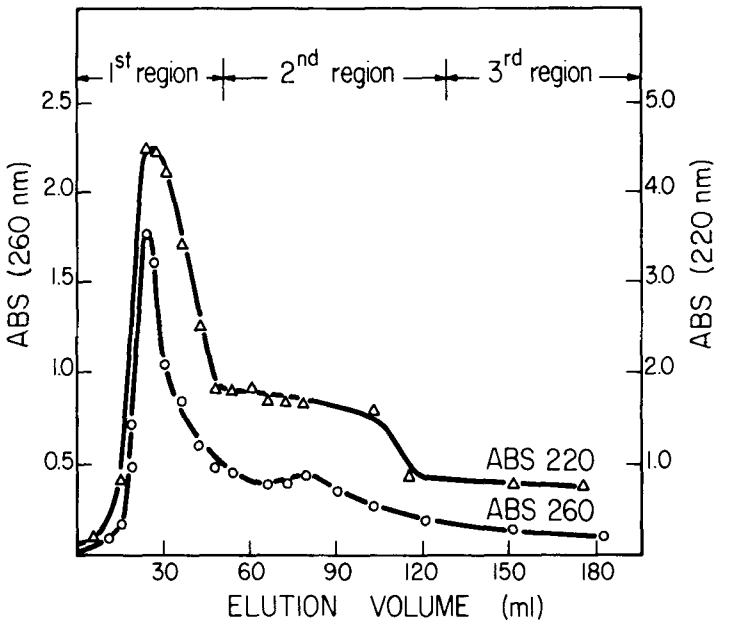

Fig. 5. Elution profile of 10 mL casein enzymatic hydrolysate. Absorbance at 220 nm (△—△) and 260 nm (○—○) measured against the eluent. For other details *see* Fig. 1.

prepared. A ten-mL sample of this solution was applied to one column. Figure 5 shows it could be fractionated into three different regions. The first one shows the highest content of peptides (Abs 220 nm) and of aromatic amino acids (Abs 260 nm).

The total mass recovery was about 88%, the first region contains about 86% of this total, mostly as peptides, with a Phe content of 1.48%. The mass contents of the other two regions are 9.40 and 4.40% respectively, with a Phe percentage of 4.09 and 19.15%.

These three regions account for about 50% of Phe initially present. Therefore, the adsorption efficiency is lower than that obtained for acid hydrolysates. The decrease in the adsorption efficiency can be caused by the presence of Trp and by the fact that Phe is present mainly linked to other amino acids in oligopeptides.

Table 5 shows the amino acid composition of these regions. The results are presented as described for Table 4.

The analysis of Fig. 5 and Table 5 shows that the first region is rich in peptides with an outstanding polar characteristic. The reapplication of this first region to a second column seems unproductive because, if some adsorption of Phe occurs, there will be a simultaneous decrease in the recovery of other amino acids.

Table 5
Percentage Composition of Regions

Amino acid nature	Region 1st, %	2nd, %	3rd, %
Polar	67.77	39.43	40.96
Nonpolar	29.93	51.07	39.12
Aromatic			
Phe	1.48	4.09	19.15
Tyr	0.82	4.90	3.63
Trp	nd	nd	nd

CONCLUDING REMARKS

The resin Amberlite XAD-4 was used to reduce Phe content of an acid hydrolysate of casein from 4.39 to 0.14%, a suitable level for dietary consumption in phenylalaninemies. The application of this technique to enzymatic hydrolysates requires special hydrolytic conditions where Phe is free or in form of small adsorptive peptides. The data obtained for the acid hydrolysate, using this simple, effective, and inexpensive technique, suggest its suitability for further scaling up studies. Variation of parameters, such as alteration of resin volume and column diameter, should lead to greater productivity.

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