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ORIGINAL RESEARCH

Experimental model for optimization of hydrophobic interaction: chromatographic purification of glucagon

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ABSTRACT: The chromatographic behavior of the components of glucagon-containing material from bovine pancreas by hydrophobic interaction on Amberlite XAD-7 was investigated. The effect of initial ionic strength and pH of the mobile phase on selectivity was quantitatively determined. The data, obtained from 56 experiments, were used for modeling and the corresponding management of the purification process. The purified glucagon is of very high quality: total impurities are not more than 1.7% as determined by analytical HPLC. The biological activity is 1.08 IU/mg, calculated for dry substance. Copyright © 1999 John Wiley & Sons, Ltd.

INTRODUCTION

Glucagon (molecular weight 3485 Da) is a small polypeptide hormone having 29 amino acids which is secreted by the A cells of the pancreatic islets and, along with insulin, which is secreted by the B cells, plays an important role for carbohydrate metabolism (Unger and Orci, 1977). The traditional process for its production is extraction from pancreas. It has been established that during the extraction and purification glucagon undergoes some proteolytic degradation. Low molecular weight degradation products, several protein-like impurities, mono- and didesamido glucagons, (des-His1) glucagon, insulin and other residues were found in glucagon batches produced from both cattle and swine pancreas (e.g. Mazzola *et al.*, 1985). For further purification, ion-exchange and gel filtration methods were applied (e.g. Cole, 1960; Stilz and Jackson, 1977; Maskalick and Anderson, 1986). Thus, the purification is a multistage process of rechromatography, with accompanying diminishing of the recovery.

In a previous paper (Angelova and Dimov, 1996) a hydrophobic interaction chromatography (HIC) step which can be applied for both separation and/or purification of glucagon has been proposed. In this paper the effects have been modeled of pH and initial salt concentration in the mobile phase on the purity of glucagon obtained by the preparative HIC stage.

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Mobile phases with high starting salt concentrations are used in HIC to induce an interaction between the protein and the matrix (e.g. Melander *et al.*, 1989; Rassi *et al.*, 1990; Szepesy and Rippel, 1992). The descending salt gradient (or step-wise elution) diminishes the hydrophobic interaction and allows the protein to be eluted from the column (e.g. Huddleston *et al.*, 1994; Gagnon and Grund, 1996).

The effect of salt concentration on stationary phase selectivity is a complex phenomenon. It appears to include the surface tension of the solution, any specific interactions between the protein and the salt ions which may not or may alter the protein structure, and the hydratation of the protein (Melander and Horvath, 1977; Fausnaugh and Regnier, 1986; O'Farrell, 1996).

The pH value of mobile phase affects the hydrophobicity of the protein itself, and hence its interaction with the hydrophobic matrix. A protein may change its conformation, as established in acid media or it may become more charged at low or high pH values and become more polar, hence less hydrophobic. Each protein reacts differently to changes in pH value.

Retention and selectivity in HIC depend also on the type of stationary phase (e.g. Miller and Karger, 1985; Rippel and Szepesy, 1994). Thus, theoretical prediction of both the effect of pH and initial salt concentration on the retention time of a given protein and on selectivity of stationary phase seems to be impossible.

Elsewhere it has been reported (Angelova and Dimov, 1996) that Amberlite XAD 7 (Rohm and Haas Company, 1981) is a suitable stationary phase for preparative HIC purification of glucagon. The composition of the mobile

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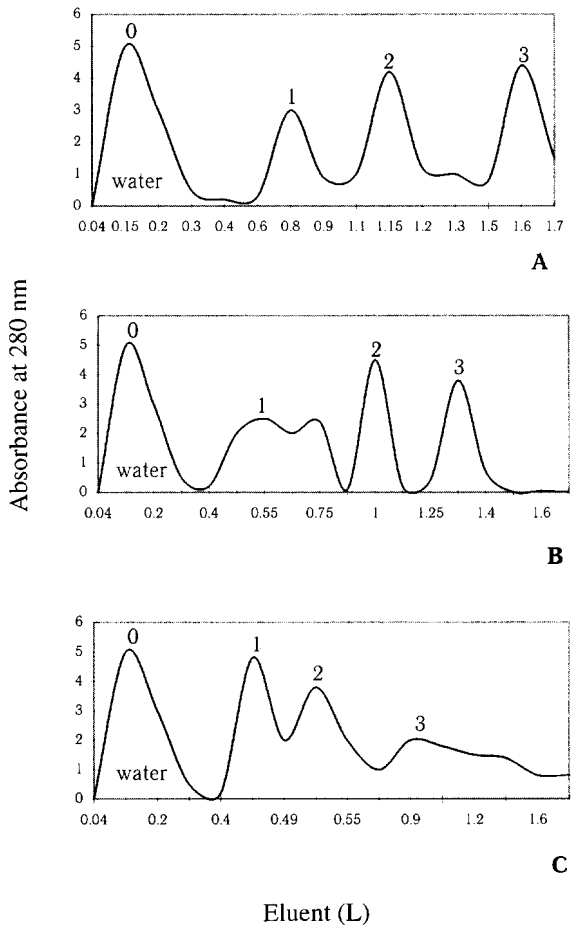

Figure 1. Effect of initial salt concentration on protein chromatographic behavior in step-wise elution. Column, Amberlite XAD 7, 3 cm i.d. × 30 cm; flow rate, 4 mL/min; temperature, 10°C; protein sample, 2 L glucagon-containing material ($E = 2.6$ at 280 nm) from bovine pancreas. In chromatogram A, the initial ionic strength of sodium chloride was 0.15 mol/L, in B it was 0.10 mol/L and in C it was 0.05 mol/L in composition with Eluent B (ethanol). Eluents B and A were mixed at various ratios (v/v): 1.5:8.5 (peak 1), 3:7 (peak 2) and 4:6 (peak 3).

phase, however, requires optimization. Therefore, for a prescribed change of the mentioned effects on glucagon purification by HIC, an experimental model is beneficial.

EXPERIMENTAL

Materials. Natural glucagon (bovine) was obtained from Calbiochem Corp. (Lucerne, Switzerland), aprotinin (protease inhibitor, trade name Trasilol) from Bayer (Leverkusen, Germany), and Amberlite XAD 7 copolymer from Rohm and Haas Chemical Company (Paris, France). Analytical standard reagent kits for radioimmunoassay of glucagon were obtained from the Scientific Research Institute of Technology of Blood Substitutes and Hormonal Substances (Moscow, Russia).

Apparatus. HPLC equipment with a Vydac 'Protein C4' column (0.46 × 15.0 cm) and glass columns (300 mm, 30 mm i.d.) was used. The eluent was monitored using a UV-2 detector, Dual Path Monitor (Pharmacia-LKB, Sweden), at 280 nm. The quantitation of PAGE (polyacrylamide gel electrophoresis) was performed on a Glinscan densitometer (Helena Laboratories, Beaumont, TX, USA).

Methods. Reversed-phase high-performance liquid chromatography (RP-HPLC): for analytical RP-HPLC, a linear gradient for the mobile phase was applied. The mobile phase consisted of water, containing 0.1% trifluoroacetic acid and acetonitrile. The concentration of the acetonitrile was increased from 25 to 35% over 25 min. The flow rate was adjusted to 1 mL/min and the UV detector was set at 220 nm.

Amino acid analysis: two samples of standard glucagon (0.80 and 1.20 μmol) and two of purified glucagon (0.80 and 1.20 μmol) were hydrolyzed in 6 mol/L HCl for 24 h at 110°C and the composition was analyzed by the amino acid analyzer AAA-Alpha Plus (Pharmacia LKB, Uppsala, Sweden). The values for tryptophan were determined in another sample after alkaline hydrolysis.

Radioimmunoassay: the method of Heding (1971) was adapted with modifications for the radioimmunoassay of glucagon. The incubation mixture (0.3 mL) contained: 4 pg ^{125}I glucagon, Trasylol (300 U/mL), 0.05 mL nonradioactive glucagon in various concentrations, and human serum albumin (1 mg/mL) in appropriate concentrations of antiserum. For dilution 0.04 mol/L sodium phosphate, pH 7.4, containing sodium chloride (6 mg/mL) was used.

The incubation was carried out at 4°C for 2 days. For precipitation, 0.6 mL ethanol was added. The mammalian crystalline glucagon standard was employed in all assays.

Polyacrylamide gel electrophoresis (PAGE): the gel electrophoresis method on a 15% polyacrylamide gel at pH 8.3 and 4.5 (e.g. Reinsfeld *et al.*, 1962; Ornstein, 1964; Jovin, 1973) was used for determination the glucagon fraction in the glucagon-containing material.

Biological assay of glucagon: the potency of glucagon was estimated by comparing its hyperglycaemic activity with the standard preparation of glucagon (British Pharmacopeia 1988).

Preparative HIC. Preparative HIC was performed on a Amberlite XAD 7 column (3 × 30 cm). The step-wise HIC was found to be a better technique than gradient elution. For more details see Angelova and Dimov (1996).

The starting glucagon-containing material was a solution obtained from industrial refuse of insulin production after ion-exchange isolation of bovine insulin. It contained about 40 mg/mL total solids, 8 mg/mL proteins and 1.5 mg/mL glucagon. Analysis of this bulk glucagon by PAGE showed high heterogeneity.

The effect of changing pH and initial salt concentration of the buffer used in step-wise elution was different for each of the proteins studied. To find the interrelations between pH and ionic strength with the obtained glucagon purity, a series of experiments was undertaken. The mobile phase for the desorption step consisted of different concentrations of sodium chloride solution, brought to different pH values using a 10% solution of ammonium hydroxide. This mobile phase was called 'eluent A'; the added ethanol was called 'eluent B'. Eluents A and B were mixed at different ratios (v/v). The desorption flow-rate was 4 ml/min.

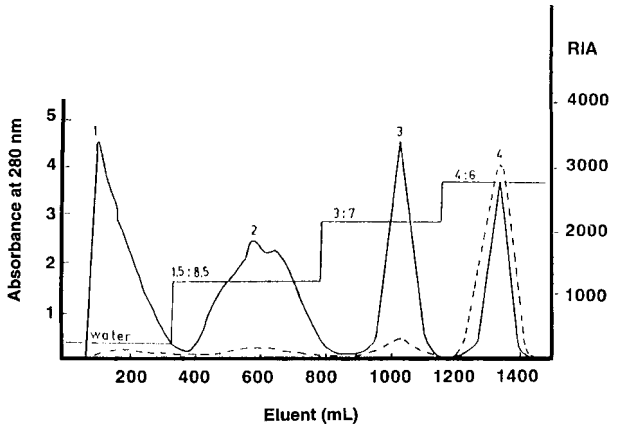

Figure 2. Hydrophobic interaction chromatography of pancreatic proteins. Conditions and elution profile of the crude glucagon on Amberlite XAD 7 step wise-elution: column, Amberlite XAD 7, 3 cm i.d. × 30 cm; temperature, 10°C; flow rate, 4 mL/min; protein sample, 2 L glucagon-containing material ($E = 2.6$ at 280 nm) from bovine pancreas. Elution with: water 320 mL; mixed buffer of ethanol, 0.1 mol/L sodium chloride, pH 10.1, (1.5:8.5) 480 mL, (3:7) 380 mL and (4:6) 300 mL. (——) Chromatogram obtained by UY monitoring at 280 nm; (-----) concentration glucagon (mg/L) monitored by RIA. 1. Polypeptides of bovine pancreas; (2) mixed fraction, containing protease inhibitor of the Kunitz (determined by PAGE, pH 4.5), glucagon-like peptides, and low molecular weight peptides; (3) insulin (determined by PAGE, pH 8.3) together with glucagon-like peptides; (4) glucagon.

RESULTS AND DISCUSSION

The study began with an examination of the importance of the selection of the initial salt cocentration for control of the purification process in HIC. The initial ionic strength had a significant effect on protein chromatographic behavior. Proteins eluting very early or very late in the step-wise elution had sharper peaks than those eluting in the middle of the step. For example, when solutions of sodium chloride were used, with concentrations 0.15 (Fig. 1A), 0.10 (Figs 1B and 2) and 0.05 mol/L (Fig. 1C) (pH 10.1–10.2), the retention time and purity of glucagon (Fig. 1, peak 3, and Fig. 2, peak 4) were different for each of the mobile phase compositions.

Further experiments were undertaken to check the influence of pH. As can be seen from Table 1, the effects of both salt and pH were different for the different groups of proteins. For example, when a solution with sodium chloride concentration 0.1 mol/L was used, the purification for glucagon depended on the pH value of the mobile phase.

Combining all results we succeeded in obtaining an adequate model, describing the surface of glucagon purity as a function of both buffer pH and NaCl concentration. The equation describing the relationship between the percentage of glucagon in the pool elution

Table 1. Effect of initial ionic strength pH on the purity of glucagon fraction in step wise elution

		Purity of separated fractions, determination by PAGE at pH 8.3 (given as %)		
H	NaCl [mol/l]	Glucagon	Desamido glucagon	Protein impurity
1	2	3	4	5
9.2	0.02	30	10	60
	0.05	26	18	56
	0.08	62	9	29
	0.10	72	8	20
	0.12	70	8	22
	0.15	40	8	52
	0.2	40	8	52
9.5	0.02	30	26	44
	0.05	40	26	34
	0.08	75	10	25
	0.10	83	9	8
	0.12	83	9	8
	0.15	50	8	42
	0.2	50	8	42
9.8	0.02	30	26	44
	0.05	40	26	34
	0.08	84	12	4
	0.10	87	9.4	3.6
	0.12	75	9	16
	0.15	70	8	22
	0.2	60	8	32
10.0	0.02	41	29	30
	0.05	41	29	30
	0.08	85	10	5
	0.10	90	9.4	0.6
	0.12	87	10	3
	0.15	80	10	10
	0.2	68	9	23
10.2	0.02	42	28	30
	0.05	42	28	30
	0.08	87	9	4
	0.10	91	8.5	0.5
	0.12	87	10	3
	0.15	77	10	13
	0.2	68	9	23
10.5	0.02	30	30	40
	0.05	26	35	39
	0.08	80	17	3
	0.10	82	16.5	1.5
	0.12	70	15	15
	0.15	68	15	17
	0.2	50	15	35
10.8	0.02	20	30	50
	0.05	23	35	42
	0.08	70	16	14
	0.10	72	16	12
	0.12	65	15	20
	0.15	60	15	25
	0.2	50	10	30
11.0	0.02	20	30	50
	0.05	23	30	47
	0.08	60	25	25
	0.10	60	16	24
	0.12	50	16	34
	0.15	40	16	44
	0.2	40	16	44

Table 2. Comparison of experimental and calculated on the basis of equation (1) values

		Quantity of glucagon in glucagon fractions, determination by PAGE at pH 8.3 (given as %)	
pH	NaCl [mol/l]	Experimental	Calculated
1	2	3	4
9.2	0.08	62	65
	0.10	72	74
	0.12	70	72
9.5	0.08	75	78
	0.10	83	85
	0.12	83	82
9.8	0.08	84	86
	0.10	87	92
	0.12	75	87
10.0	0.08	85	88
	0.10	90	93
	0.12	87	87
10.2	0.08	87	88
	0.10	91	94
	0.12	87	85.5
10.5	0.08	80	84
	0.10	82	86
	0.12	70	78
10.8	0.08	70	74
	0.10	72	75
	0.12	65	65.5
11.0	0.08	60	64
	0.10	60	64
	0.12	50	54

fraction (column 3, Table 1) and pH and initial salt concentration is:

$$y = -3206(\pm 467) + 610(\pm 88) \cdot x_1 - 29.3(\pm 4.3) \cdot x_1^2 + 4944(\pm 20.1) \cdot x_2 - 13125(\pm 70.9) \cdot x_2^2 - 234(\pm 1.4) \cdot x_1 \cdot x_2 \quad (1)$$

where y is percentage of glucagon in pool elution fraction, x_1 is the pH value of mobile phases and x_2 is initial salt concentration (mol/L) of mobile phases.

All of the parameters in this quadratic equation are statistically significant. The correlation coefficient is high enough ($r = 0.970$) and the relative error is sufficiently low—2.2%. The comparison of experimental and calculated values for glucagon content is presented in Table 2.

The experimental surface is depicted in Fig. 3. The strong dependence on both pH and salt concentration is well illustrated. There is a region at about pH = 10.0 in wich the HIC purification is relatively stable against small changes in NaCl concentrations. As a conclusion, hydrophobic interaction is affected more strongly by the change in pH than by the change in initial ionic strength.

The experimental model gives the following intervals for stable optimum conditions of purification: eluent A with 0.1 ± 0.005 mol/L concentration of sodium chloride

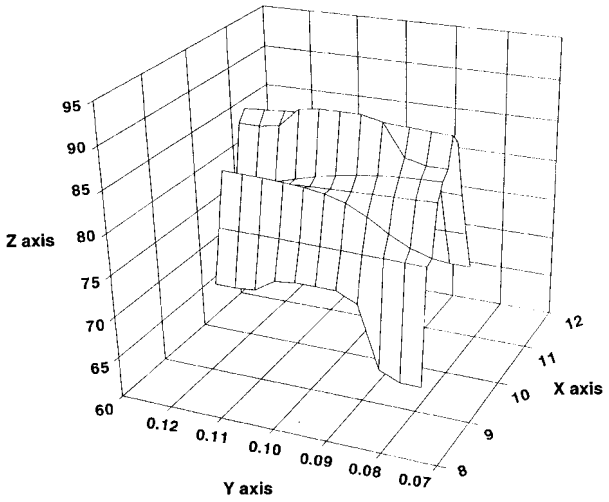

Figure 3. Three-dimensional drawing of all experimental data given in Table 1. *X* axis—pH, *Y* axis—salt concentration, *Z* axis—glucagon content.

solution, brought to pH 10.1 ± 0.05 using a 10% solution of ammonium hydroxide. Eluent B is ethanol (750 g/L). Eluent B and eluent A were mixed at ratios (v/v) 1.5:8.5, 3:7 and 4:6 correspondingly in the step-wise elution. Such conditions, which we assessed as mild, permitted recovery of glucagon in a biologically active form. The purified glucagon, obtained at the optimum conditions of the process was assayed by analytical HPLC and its purity was above 98.3%.

After crystallization of the purified glucagon, an activity of 1.08 IU/mg, calculated for dry substance, was obtained. The amino acid composition (Table 3) is in good agreement with that of the standard glucagon.

Table 3. Amino acid composition of purified glucagon and standard glucagon

Amino acid	Theoretical	Standard glucagon	Observed glucagon
Asp + Asn	4	4.0	4.10
Thr	3	2.8	3.03
Ser	4	3.7	3.75
Glu (Gln)	3	3.1	3.03
Gly	1	1.0	1.07
Ala	1	1.0	0.94
Val	1	0.9	0.95
Met	1	1.0	1.07
Leu	2	2.01	2.1
Tyr	2	2.0	1.95
Phe	2	2.0	1.98
Lys	1	1.1	0.97
His	1	1.0	0.95
Arg	2	2.0	1.91
Trp	1	1.1	1.05
	29		

The values are not corrected for partial destruction by 24 hr hydrolysis.

CONCLUSION

After choosing Amberlite XAD 7 as stationary phase for HIC purification of glucagon, two mobile phase factors, namely pH and initial NaCl concentration, can be optimized using an experimental model. This determines the optimum domain and allows better management of the process.

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