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Amyloidogenicity and Cytotoxicity of Recombinant Mature Human Islet Amyloid Polypeptide (rhIAPP)*

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Pancreatic amyloid plaques formed by the pancreatic islet amyloid polypeptide (IAPP) are present in more than 95% of type II diabetes mellitus patients, and their abundance correlates with the severity of the disease. IAPP is currently considered the most amyloidogenic peptide known, but the molecular bases of its aggregation are still incompletely understood. Detailed characterization of the mechanisms of amyloid formation requires large quantities of pure material. Thus, availability of recombinant IAPP in sufficient amounts for such studies constitutes an important step toward elucidation of the mechanisms of amyloidogenicity. Here, we report, for the first time, the successful expression, purification and characterization of the amyloidogenicity and cytotoxicity of recombinant human mature IAPP. This approach is likely to be useful for the production of other amyloidogenic peptides or proteins that are difficult to obtain by chemical synthesis.

Deposition of proteins as insoluble amyloid aggregates in tissues is a common feature in a wide range of important human diseases, including Alzheimer's disease, type II diabetes, and the transmissible spongiform encephalopathies (1). Proteinaceous fibrils are central components of the amyloid deposits, being typically composed of linear, unbranched, and non-covalent aggregates of a single polypeptide chain arranged in crossed β-pleated sheets (2). By mechanisms that are still incompletely understood, amyloid deposition leads to cell dysfunction and death, and, ultimately, to severe tissue damage in amyloid diseases.

Islet amyloid polypeptide (IAPP)[1] or amylin is a 37-amino acid peptide produced by pancreatic islet β-cells (3). IAPP is synthesized, packaged within secretory granules and co-secreted with insulin by the islet β-cells in response to elevations in plasma glucose levels (4). The *IAPP* gene, located in chromosome band 12p12.3-p12.1, is transcribed as a 1.5-kb message that codes for an 89-amino acid precursor protein (pro-IAPP), which is subsequently processed to yield the mature polypeptide (5). Previous studies have suggested that proprotein convertases PC3 (also known as PC1) and PC2 are responsible for processing the prohormone to the active secreted form of IAPP (6). Although it has been proposed that PC2 is primarily responsible for IAPP processing within secretory granules (7), both PC3 and PC2 are likely to be responsible for pro-IAPP processing (8). Soluble IAPP plays important roles in carbohydrate metabolism and is currently considered to be the third pancreatic islet hormone active in glucose homeostasis (4, 9).

Pancreatic amyloid plaques formed by IAPP are present in more than 95% of type II diabetes mellitus patients, and their abundance correlates with the pathological sequelae of the disease (10). Transgenic mice expressing human IAPP (h-IAPP) exhibit a diabetic phenotype associated with the deposition of h-IAPP in amyloid form (11). *In vitro*, IAPP has a strong tendency to aggregate into amyloid fibrils, and has recently been described as the most amyloidogenic polypeptide known (12). The molecular determinants of the aggregation of IAPP are not yet fully understood. *In vitro*, IAPP aggregation follows a kinetics that is consistent with a nucleation-dependent polymerization mechanism (5).

Detailed characterization of the molecular and conformational properties of amyloidogenic peptides and of the mechanisms of amyloid formation requires large quantities of pure material. Thus, chemical synthesis or recombinant expression and purification of IAPP in sufficient amounts for such studies constitute important steps toward the elucidation of the mechanisms of amyloidogenicity. However, the chemical synthesis of amyloidogenic peptides is often difficult (13) and production of most of them, including IAPP, in the recombinant form has not yet been achieved. A recent study by Krampert *et al.* (5) reported the bacterial expression of recombinant human proIAPP fused to a histidine tag, but that fusion product was found to be markedly less amyloidogenic than native IAPP. Another recent report described the expression and purification of a recombinant MBP (maltose-binding protein)-IAPP fusion protein, fail-

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[1] The abbreviations used are: IAPP, islet amyloid polypeptide; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; IPTG, isopropyl-1-thio-β-D-galactopyranoside; TFE, 2,2,2-trifluoroethanol; ThT, thioflavin T; rhIAAP, recombinant mature human IAAP; TEM, transmission electron microscopy; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; Trx, thioredoxin.

ing in the attempts to express the intact, mature human IAPP (14). Here, we report the successful expression, purification and characterization of human mature IAPP utilizing the LacI-T7 RNA polymerase-based heterologous expression system for *Escherichia coli*. The solution conformation and the amyloidogenic and cytotoxic potentials of recombinant mature human IAPP (rhIAPP) were characterized using a variety of biochemical and biophysical methods, including light scattering, thioflavin T (ThT) fluorescence measurements, transmission electron microscopy (TEM), and cytotoxicity assays using primary cultures of human β-cells. The approach described here is likely to be useful for the recombinant expression of other amyloidogenic peptides or proteins that are difficult to obtain by chemical synthesis.

EXPERIMENTAL PROCEDURES

Reagents—The pET bacterial expression system used is from Novagen Corp. (Madison, WI). Primers were synthesized by Invitrogen (São Paulo, Brazil). Molecular biology enzymes and reagents were from Amersham Biosciences (Uppsala, Sweden), Invitrogen, Novagen, and Fermentas Ltd. (Vilnius, Lithuania). Thioflavin T, enterokinase, CMRL cell culture medium, and all other reagents were from Sigma. Human synthetic IAPP was purchased from Bachem Inc. (Torrance, CA). Predistilled water was filtered and deionized through a Millipore purification system.

Cloning of N-tagged Human Mature IAPP—A 151-bp DNA fragment containing the coding sequence for the mature IAPP peptide was amplified by reverse transcription polymerase chain reaction (RT-PCR) from 5 μg of total RNA from human pancreatic islets purified at the Human Islet Unit of the University of São Paulo. Total RNA was purified after cell lysis with guanidine thiocyanate/β-mercaptoethanol followed by ultracentrifugation on a cesium chloride cushion (41). Reverse transcription was carried out with SuperScript II (Invitrogen) according to the manufacturer's instructions. PCR amplification was performed with Platinum Taq High Fidelity (Invitrogen) using an initial denaturing step at 95 °C for 1 min, followed by 35 cycles of incubation at 95 °C for 30 s, 55 °C for 30 s, 68 °C for 15 s, and a final extension step of 5 min at 68 °C. Forward and reverse primers corresponding to the 5′- and 3′-ends of the human mature IAPP coding sequence (IAPP-F: 5′-AGA TCT GGG TAC **CGA CGA CGA CGA CAA** GAA ATG CAA CAC TGC CAC AT-3′; IAPP-R: 5′-GTC GAC TCA ATA TGT ATT GGA TCC CAC GTT GGT AG-3′) were designed to contain BglII and SalI restrictions sites (underlined) for directional and in-frame cloning, as well as a stop codon inserted in-frame after the last residue (italic). An enterokinase (Ek) cleavage site coding sequence (in bold) was also added to the forward oligo immediately before the first amino acid residue of IAPP in order to allow cleavage of mature IAPP from the vector-encoded protein tag.

The resulting 151-bp PCR product generated was cloned into the pUC 18 plasmid vector and ligated to the pET32a vector, which was previously digested with the same enzymes and dephosphorylated. Therefore, in this cloning strategy, the Ek site from the vector was removed and replaced by the Ek site-containing PCR insert. Therefore, IAPP was expressed as an N-fusion protein with the vector-encoded HT-TRX-S tag. The ligated product was electroporated into competent DH10B *E. coli*. A recombinant clone (pET32a-IAPP) was isolated and characterized by restriction digestion and automated sequencing.

Bacterial Expression and Purification—The pET32a-IAPP plasmid containing the IAPP fusion protein was used for transformation of competent BL21(DE3)pLysS cells by electroporation. These cells were grown at 37 °C in a bioreactor in 1 liter of Super Broth medium (20 g of tryptone, 12 g of yeast extract, 5 ml of NaOH, 5 g of NaCl) containing ampicillin (150 μg/ml). When the absorbance at 600 nm reached 0.6 ($\sim 1.7 \times 10^8$ cells/ml), isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM. After 6 h of induction at 37 °C, the medium was centrifuged at 5,000 $\times$ *g* at 4 °C for 30 min, and the pellet was resuspended in 500 ml of 20 mM Tris-HCl, 500 mM NaCl, 1 mM imidazole buffer, pH 7.4. Cells were lysed using an ultrasonic homogenizer (Vibracell 72412; Bioblock, Illkirch, France) at 20 kHz, using a 19-mm probe, and ten 1-minute pulses on ice, with 2-min intervals between pulses. The material was kept on ice throughout the lysis step. The suspension was centrifuged at 18,000 $\times$ *g* at 4 °C for 40 min in order to pellet the inclusion bodies containing IAPP. The pellet was then resuspended in 250 ml of the same buffer containing 6 M urea and incubated overnight at 4 °C for solubilization of the inclusion bodies. To remove any remaining insoluble material, the sample was centrifuged at 39,000 $\times$ *g* at 4 °C for 20 min. The clear supernatant was collected, filtered through a 0.45-μm membrane and used for IAPP purification on an immobilized metal-ion affinity chromatography column, using Ni^{2+}-nitrilotriacetic acid (Ni-NTA) agarose (Amersham Biosciences) in the presence of 6 M urea.

The Ni-NTA column was washed with 3 bed volumes of the buffer described above in the absence of urea followed by three washes with the same buffer plus 6 M urea, using a flow rate of 1 ml/min. The column was then loaded with 300 ml of solubilized IAPP at a flow rate of 0.25 ml/min. After loading, the column was washed with 3 bed volumes of washing buffer (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 7.4) plus 6 M urea. Urea was slowly removed using a discontinuous gradient (from 6 to 0 M urea) in the same washing buffer. The IAPP fusion protein was cleaved with enterokinase within the column to remove the thioredoxin and His tags. Enterokinase (1 unit) was loaded onto the column in the cleavage buffer containing: 10 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.0, and the cleavage reaction was carried out for 18 h at 25 °C. The purified rhIAPP was eluted with a solution of 50% (v/v) 2,2,2-trifluoroethanol (TFE) in the same cleavage buffer. The concentration of pure rhIAPP fractions was determined from the UV absorbance at 280 nm, considering a molar extinction coefficient of 1.330 M^{-1} cm^{-1}.

Electrophoresis Systems—Analysis of fractions obtained at various steps of the expression and purification process was carried out using SDS-PAGE or Tricine electrophoresis (20). Coomassie Brilliant Blue R-250 or silver staining was used to visualize the proteins bands.

Matrix-assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Analysis—Aliquots of purified rhIAPP were analyzed on an ABI 4700 Analyzer with TOF-TOF Optics (Applied Biosystems, Framingham, MA) at the National Synchrotron Laboratory (LNLS). 3 μl of purified peptide solution ($\sim$5 pmol) were mixed with 6 μl of matrix solution (5 mg of α-cyano-4-hydroxy-cinnamic acid (Aldrich) in 50% acetonitrile, 0.1% trifluoroacetic acid) and placed on the sample plate to dry. Calibration and mass measurements of the rhIAPP samples were carried out in the linear mode. Calibration of the instrument in the low molecular mass range was carried out using Calibration Mixture 3 from the Peptide Mass Standard Kit number 3 (Applied Biosystems).

Peptide Aggregation—Stock IAPP solutions were maintained in 50% TFE. Aggregation was triggered by diluting aliquots of the stock solution in buffer containing 100 mM Tris-HCl, 150 mM NaCl, pH 7.4 (prefiltered on a 0.45-μm filter), to a final concentration of 2.5 μM IAPP (residual TFE concentration of 1%).

For cytotoxicity assays, lyophilized synthetic IAPP (Bachem) was resuspended to a final concentration of 3 mM in 50% TFE immediately prior to the addition to the cell cultures. Alternatively, the fresh stock solution of rhIAPP was diluted to a final concentration of 60 μM in the cultures. The residual TFE concentration in the culture was $\leq$ 1.2% (v/v) in both conditions. Control assays showed no effects of these low concentrations of residual TFE on cell viability.

Fluorescence Measurements—IAPP aggregation and formation of amyloid fibrils were determined by right-angle light scattering (LS) and thioflavin T (ThT) fluorescence measurements using an ISS-PC spectrofluorometer (Hitachi F-4500). The samples were incubated in a dry bath at 37 °C for the duration of the experiment (up to 120 h). LS was measured at 500 nm. ThT was added to a final concentration of 5 μM and the fluorescence signal (excitation at 450 nm) was recorded between 482 and 487 nm (42, 43). Nonspecific background fluorescence was subtracted from the samples by using appropriate blanks not containing protein.

Cell Culture and Cytotoxicity Assays—Purified human islet cells were obtained at the Human Islet Unit (University of São Paulo, Brazil) by collagenase digestion according to the Edmonton protocol (44). Islet cells were plated onto 48-well plates at a density of 5×10^4 cells per well in CMRL medium supplemented with 10% fetal calf serum, 100 units/ml of penicillin and 100 units/ml of streptomycin. After 24 h, the plates were washed twice with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2PO_4, pH 7.4) and the concentration of serum was lowered to 0.5% before addition of IAPP to the cultures at the concentrations indicated under "Results." Untreated control cultures received the same volume of phosphate-buffered saline. The cultures were further incubated at 37 °C, 5% CO_2 for 72 h, and then carefully washed with phosphate-buffered saline to remove the dead cells, collected by trypsinization, fixed in 3.7% formaldehyde and counted in a CELM CCS 530 electronic cell counter (CELM, São Paulo, Brazil). The data were statistically evaluated using analysis of variance and Student's *t* test. A p value < 0.05 was considered significant.

Electron Microscopy (EM)—Samples of IAPP which had undergone

aggregation for 72 h were applied to Formvar-coated nickel grids for 2 min and dried. These samples were negatively stained with 2% (v/v) uranyl acetate for 45 s and washed with a drop of water. The samples were air-dried and examined on a Zeiss 900 electron microscope operated at 80 kV.

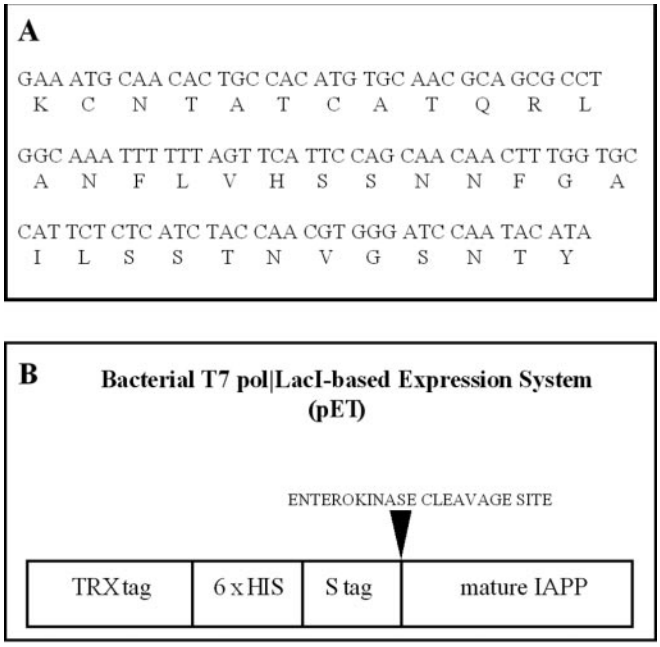

FIG. 1. **Schematic representation of the sequence and fusion construct of human mature IAPP.** *Panel A*, cDNA and amino acid sequences of IAPP. *Upper lines* represent the nucleotides and *lower lines* are the corresponding amino acids. *Panel B*, scheme of the expressed fusion protein. Mature IAPP was expressed as an N-fusion to the TRX-HIS-S tag region of the expression vector. The enterokinase cleavage site is indicated.

RESULTS

Design of the Recombinant Human IAPP—We used the pET *E. coli* expression system and the BL21(DE3)pLysS-pET host strain to produce mature IAPP (Fig. 1). This system has been shown to efficiently suppress unwanted expression of potentially toxic proteins and to produce high levels of recombinant protein products in an inducible fashion (15). For this purpose, RT-PCR was employed to generate a cDNA fragment encoding the mature IAPP (Fig. 2). Modifications at the 5′-end of the PCR oligonucleotides were introduced for in-frame and directional subcloning of the amplified fragment and to allow proteolytic cleavage of the recombinant fusion protein. BglII and SalI sites were introduced in the forward and reverse oligonucleotides, respectively, in order to allow for in-frame cloning of the fragment with the vector-encoded HT-TRX-S tag. In addition, the enterokinase cleavage site was removed from the vector, and another cleavage site was inserted into the forward oligo in-frame and juxtaposed to the first IAPP amino acid (Fig. 1*B*). This prevented the addition of a dipeptide tail to the N-terminal of IAPP that would otherwise be inserted in case the vector-encoded Ek site was used instead of the Ek site added to the primer. High fidelity RT-PCR using total RNA extracted from human pancreatic islets yielded the cDNA in a clear, single band of 151 bp, as expected (Fig. 2*B*). This fragment was cloned into the pUC18 vector and subcloned into the pET32a bacterial expression vector. The IAPP insert was restriction digested and subjected to automated DNA sequencing of both strands of both the vector and the inserted primers in order to check the integrity of the cloned sequence and the correct reading frame relative to the vector encoded protein tag (data not shown).

Thioredoxin (Trx) has been successfully used as a fusion moiety to express insoluble recombinant proteins in *E. coli* (16). Previous reports have shown that fusion to Trx dramatically increases the solubility of heterologous proteins synthesized in

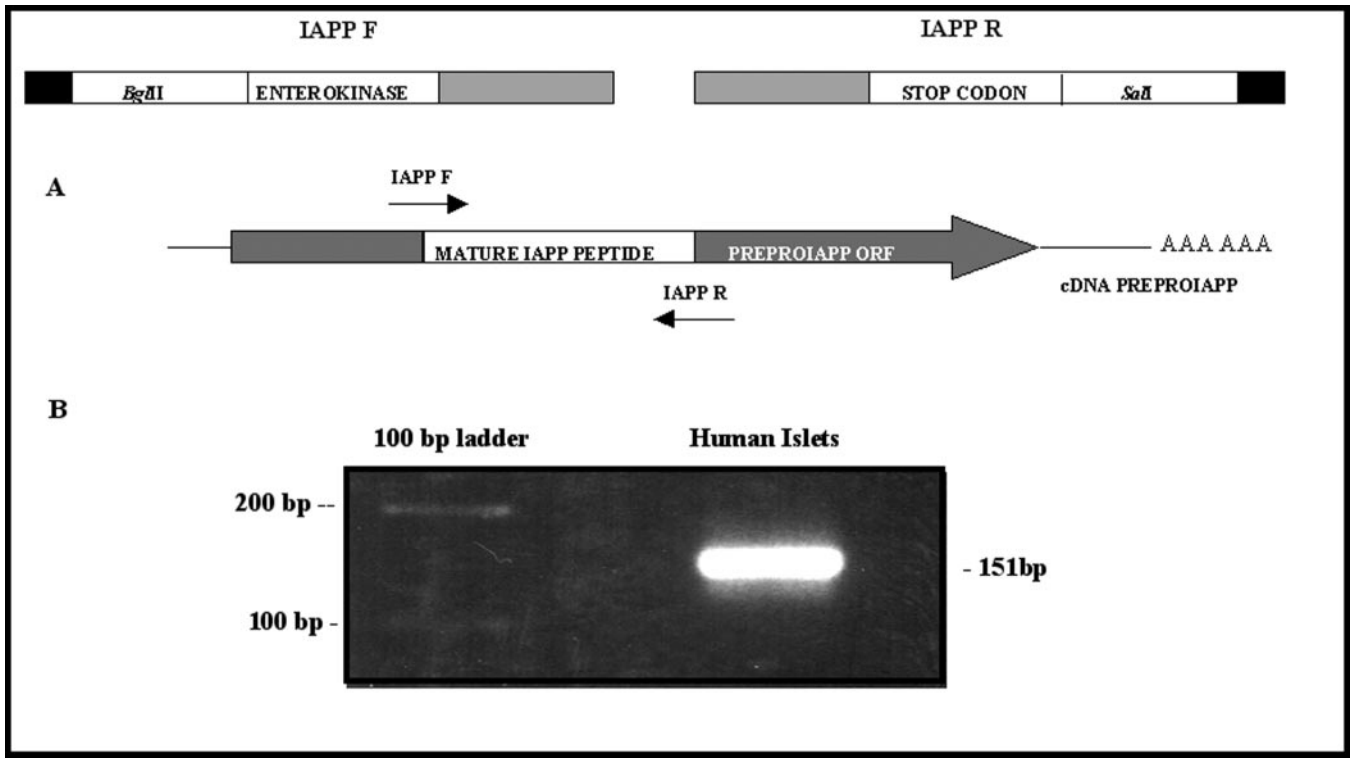

FIG. 2. **Cloning by RT-PCR of the cDNA for mature human IAPP.** *Panel A*, schematic representation of the cloning strategy showing the two primers for amplification of the coding region for mature IAPP. Codons for insertion of an enterokinase cleavage site, and BglII/SalI restriction enzyme sites for in-frame cloning of the cDNA are indicated. *Panel B*, RT-PCR product resulting from the amplification of IAPP from human islets cDNA using the above-mentioned primers. RT-PCR amplification yielded a single band of 151 bp.

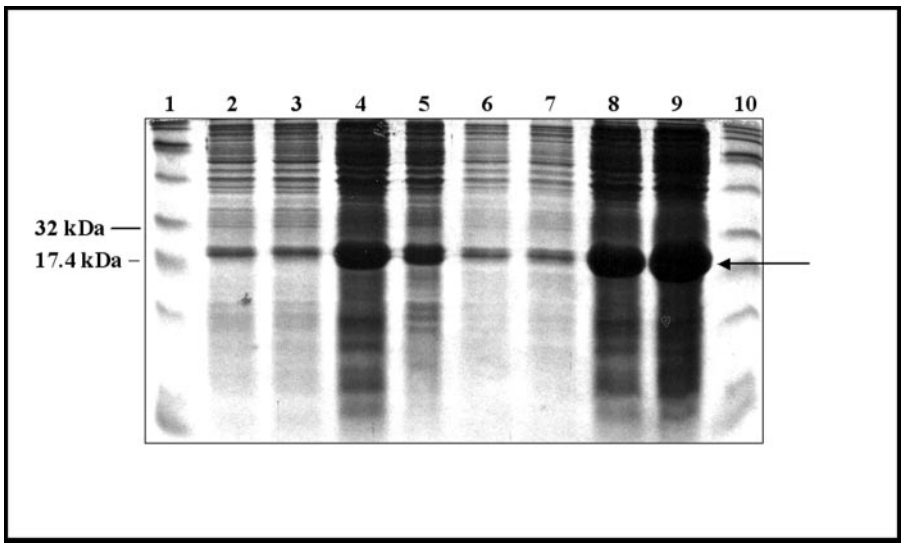

FIG. 3. **SDS-PAGE analysis of the expression of recombinant human IAPP fusion protein.** Electrophoresis was performed using an 18% acrylamide gel, and the proteins were stained with Coomassie Brilliant Blue R-250. *Lanes 1* and *10*, molecular mass markers. *Lanes 4* and *8*, crude extracts from two different bacterial cultures after 6 h of induction with 0.4 mM IPTG. *Lanes 5* and *9*, crude extracts from two different bacterial cultures after 6 h of induction with 1 mM IPTG. *Lanes 2*, *3*, *6*, and *7*, crude extracts from two different non-induced bacterial cultures. A 10-fold excess of protein was applied in *lanes 4*, *5*, *8*, and *9* compared with *lanes 2*, *3*, *6*, and *7*. The band corresponding to the IAPP fusion protein is indicated by a *black arrow* on the *right-hand side* of the gel.

E. coli, with Trx fusion proteins usually accumulating to high levels (16). In an attempt to improve the solubility and the rate of expression of IAPP, a Trx tag was included in the fusion protein, in addition to a hexahistidine (His) tag used to facilitate purification (Fig. 1). Both fusion tags could then be removed by enterokinase cleavage, resulting in full-length IAPP. The S-fragment of RNaseA (S) tag was not utilized in this study. The recombinant fusion protein has a molecular mass of about 17 kDa; the tag region corresponds to 13.2 kDa and mature IAPP is ~3.9 kDa in mass.

Expression and Purification of Recombinant Human IAPP—In order to optimize protein expression, we induced the bacterial cultures with two different concentrations of IPTG for 6 h (Fig. 3). No significant difference was found using 0.4 mM or 1 mM IPTG, and, therefore, the latter concentration was employed in subsequent experiments. With both IPTG concentrations, a prominent protein band representing the main component in the bacterial extracts was found in the induced cultures.

As might have been expected for the recombinant expression of an amyloidogenic protein, rhIAPP formed inclusion bodies. SDS-PAGE analysis (Fig. 3) showed the expected protein band only when cell lysis was carried out under denaturing conditions. When lysis was carried out under non-denaturing conditions, a pellet consisting of inclusion bodies was obtained. The pellet was washed three times with lysis buffer to remove cell debris and other impurities. After washing, the inclusion bodies were solubilized by urea, as described by Ramos *et al.* (17). The effectiveness of solubilization of inclusion bodies increased proportionally to the urea concentration (data not shown). The inclusion bodies were routinely solubilized in 6 M urea and clarified by ultracentrifugation and filtration.

The lysates were purified by immobilized metal-ion affinity chromatography, using Ni-NTA agarose under denaturing conditions. It is widely known that the affinity between proteins and metal ions under denaturing conditions is usually lower (18). Thus, in order to optimize the interaction of the solubilized protein with the column, the flow rate was decreased in this step. This approach was useful to increase the purification yield in the presence of urea.

Because of the high amyloidogenicity of IAPP (19), we performed the enterokinase cleavage step directly on the column. To this end, urea was removed by washing the column with a discontinuous gradient of decreasing urea concentrations (from 6 to 0 M). Previous tests showed that the optimal conditions of temperature and time of cleavage to avoid proteolytic degradation and to provide the best yield of IAPP were 18 h of incubation at 25 °C with 1 unit of enterokinase. Cleaved IAPP was then eluted from the column with a 50% solution of TFE in cleavage buffer. Using these procedures, the yield of rhIAPP obtained was ~10 mg of pure protein per liter of bacterial culture, which we consider to be quite significant considering that IAPP is a very hydrophobic and amyloidogenic protein.

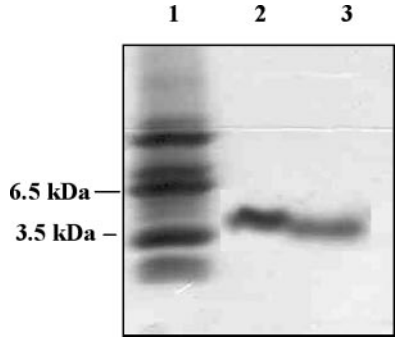

FIG. 4. **Tricine SDS-PAGE analysis of synthetic and purified recombinant human IAPP.** *Lane 1*, ultra low range molecular mass markers for SDS-PAGE. *Lane 2*, synthetic IAPP. *Lane 3*, rhIAPP eluted from the NI-NTA column after enterokinase cleavage. The gel was stained with Coomassie Brilliant Blue.

Fractions of purified rhIAPP were analyzed by SDS-PAGE using Tricine as the trailing ion in the presence of 6 M urea (20). This analysis revealed the expected band of ~4 kDa (Fig. 4).

Characterization of the Recombinant Human IAPP—Samples of commercial IAPP obtained by solid-phase synthesis and of our purified rhIAPP were analyzed by MALDI-TOF/TOF MS (Fig. 5). For synthetic IAPP, an ion ($M+H^+$) of 3,902 Da was obtained (Fig. 5*A*), and for rhIAPP a major component ($M+H^+$) of 3,903 Da was also determined (Fig. 5*B*), corresponding to full-length IAPP. Interestingly, the rhIAPP samples also contained minor lower MW peaks in addition to the main IAPP

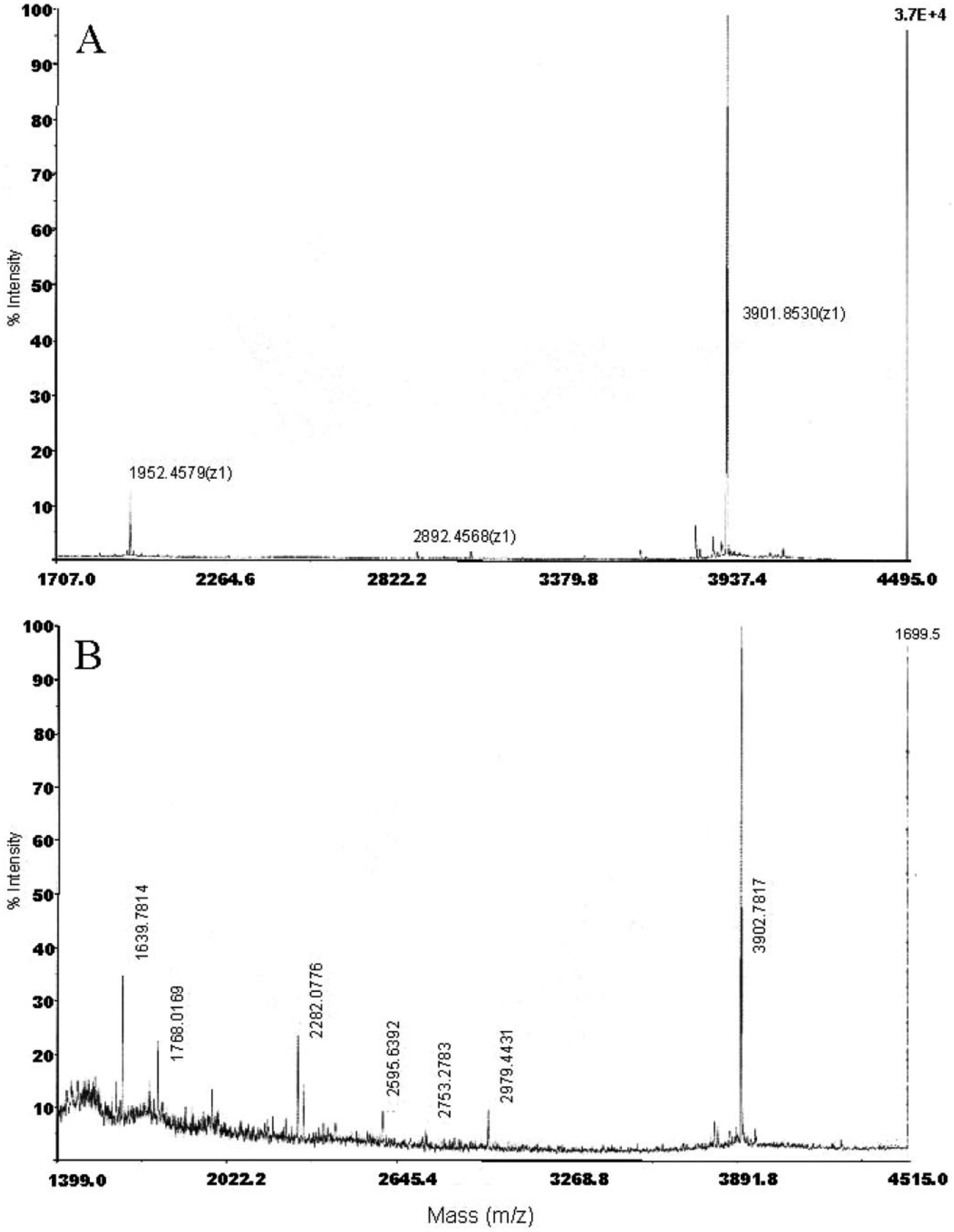

FIG. 5. **MALDI-TOF/TOF mass spectra of synthetic (*panel A*) and recombinant (*panel B*) human IAPP.**

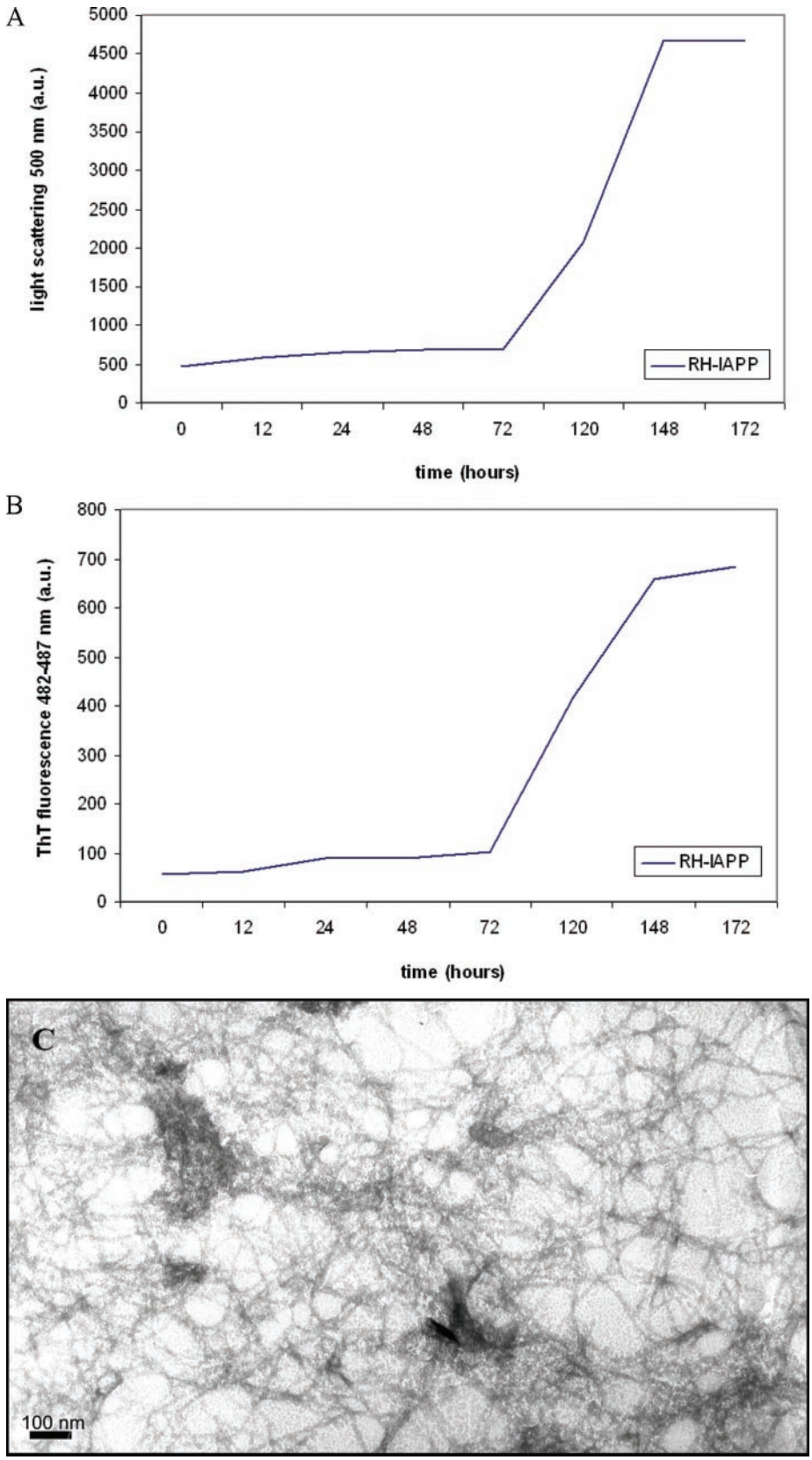

FIG. 6. **Amyloidogenicity of rhIAPP.** Samples (2.5 μM rhIAPP) were incubated at 37 °C in 100 mM Tris-HCl, 150 mM NaCl, pH 7.4, and aggregation was monitored by light scattering, ThT fluorescence measurements and electron microscopy. *A*, light scattering measurements at 500 nm for up to 172 h of aggregation of rhIAPP. *B*, ThT fluorescence emission in the 482–487-nm wavelength range for up to 172 h of rhIAPP aggregation. *C*, TEM of fibrillar aggregates of rhIAPP formed at 120 h of aggregation. *Scale bar* corresponds to 100 nm. Data are from a representative experiment of a total of four experiments.

peak. MS/MS analysis of the lower molecular mass ions showed that they correspond to peptide fragments derived from the full-length IAPP molecule (data not shown). Longer incubation periods with enterokinase led to increased amounts of the low molecular mass fragments (data not shown), whereas proteolytic treatment for 18 h or less led to reduced peptide fragmentation in the samples (Fig. 5*B*).

Previous reports have shown that human IAPP readily aggregates into amyloid fibrils in aqueous medium (3, 12, 21, 22). In order to investigate its aggregation behavior, samples of purified recombinant human IAPP were incubated for different periods of time at 37 °C in buffer and were analyzed by light scattering and ThT fluorescence measurements. The results show that amyloid fibrils are formed, as indicated by the in-

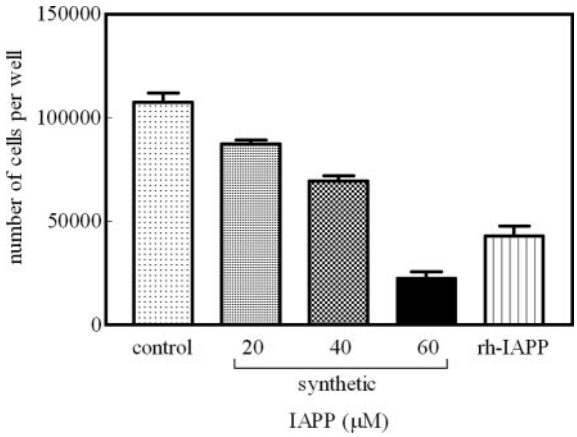

FIG. 7. **Cytotoxicity of rhIAPP and synthetic IAPP.** The samples of IAPP were added to primary cultures of pancreatic islet cells at the indicated concentrations, and cell viability was assessed by cell counting after 72 h of incubation, as described under "Experimental Procedures." rhIAPP was used at a fixed concentration of 60 μM. Data represent means ± S.D. from four independent experiments. Statistical evaluation using one-way analysis of variance Tukey's test (GraphPad Prism) indicated lack of statistically significant difference ($p > 0.05$) in the comparison between the cytotoxicities of 60 μM synthetic and recombinant IAPP.

crease in light scattering (Fig. 6*A*) and by the characteristic increase in thioflavin T fluorescence (Fig. 6*B*) as a function of time. Control measurements with the non-fibrillogenic synthetic rat IAPP showed no increase in ThT fluorescence or light scattering (data not shown).

In order to confirm the amyloidogenicity of rhIAPP, we subjected previously aggregated samples to transmission electron microscopy analysis. The results show that abundant fibrils of characteristic amyloid morphology are formed upon aggregation of rhIAPP, as illustrated in Fig. 6*C*.

The toxicity of IAPP to pancreatic β cells is well documented (9, 23, 24). In order to characterize the cytotoxicity of recombinant IAPP, human islet cells were incubated for 72 h in the presence of synthetic or rhIAPP. Addition of 60 μM rhIAPP led to a 60% decrease in cell number when compared with control cultures, in good agreement with the results obtained with synthetic IAPP (Fig. 7). This provides clear evidence of the cytotoxicity of rhIAPP.

DISCUSSION

Islet amyloid polypeptide (IAPP) is among the most amyloidogenic peptides known. For this reason, the chemical synthesis of this peptide by solid phase synthesis has been reported to be difficult (12, 25, 26). Since efficient methods for recombinant expression of proteins have become increasingly available (16, 27), the recombinant expression of polypeptides that, by length, are still within the reach of solid phase synthesis methods, has been more widely applied.

In particular, Trx-tagged fusion proteins have been successfully expressed with good yields, being specially convenient for the expression and purification of weakly soluble proteins (28, 29). Previous examples of recombinant expression of amyloid polypeptides as fusion constructs with soluble protein moieties include the expression of the Aβ peptide as a Trx-His-tagged construct (30) and the expression of the Huntington's disease protein as a GST-HD exon 1 fusion protein (31, 32). Here, we have described, for the first time, the recombinant expression and purification of human IAPP.

To verify that intact and mature rhIAPP was obtained, MALDI-TOF/TOF MS analysis of the purified protein was employed (Fig. 5). Due to its mass resolution, sensitivity, and speed of analysis, this technique has become the method of choice for the analysis of proteins and peptides. MS analysis showed that the Ek cleavage designed to proceed C-terminally of the recognition sequence $(Asp)_4$-Lys in Trx-His-IAPP was specific. However, upon incubation of the fusion protein with Ek for longer periods of time, we also observed the formation of lower molecular mass cleavage products. These by-products corresponded mainly to sequence stretches 1–15 and 16–37 of IAPP, which are likely to have resulted from Ek cleavage at the Phe^{15}–Leu^{16} amide bond, as previously pointed out (5). The similarity of the Asp^{14}-Phe^{15}-Leu^{16} sequence to the Ek recognition site $(Asp)_4$-Lys may account for the observed nonspecific cleavage (5). The other minor cleavage products of IAPP of $(M+H^+)$ masses of 1,768, 2,596, 2,753, and 2,980 Da may have been caused by the prolonged cleavage period at 37 °C that was applied in order to increase the yield of rhIAPP. In addition, a 1-Da difference was observed between the non-amidated recombinant product and the amidated synthetic polypeptide, as could have been anticipated (Fig. 5).

Since removal of the His-Trx-tags from the fusion protein might lead to a decrease in protein solubility and an increase in the amyloidogenic potential of IAPP, we chose to perform the enterokinase cleavage step while the fusion protein was still bound to the Ni-NTA column. Once the tag had been removed, elution of IAPP was carried out with buffer containing 50% trifluoroethanol. TFE is known to destabilize hydrophobic interactions within polypeptide chains and to stabilize local hydrogen bonds between residues, which are close to each other in the amino acid sequence, particularly those forming α-helix and β-hairpins (33). Nevertheless, visual inspection of the eluate after a few days revealed the presence of insoluble material, in agreement with the high amyloidogenicity of IAPP. Indeed, the amyloidogenic nature of the recombinant IAPP was confirmed by light scattering and ThT fluorescence measurements. Additional confirmation of the capacity of rhIAPP to aggregate into fibrils of typical amyloid morphology was provided by electron microscopy studies (Fig. 6*C*).

A direct causal relationship between amyloid aggregation of IAPP and cell death has not yet been demonstrated (19). Various amyloidogenic peptides, including IAPP, have been found to be cytotoxic to a variety of cell types (9, 23), suggesting that amyloid fibrils, protofibrils or oligomeric aggregates are cytotoxic. In agreement with previous results obtained with synthetic IAPP, rhIAPP was markedly cytotoxic to primary cultures of human islets cells (Fig. 7).

IAPP belongs to the calcitonin-gene related peptides (CGRPs) family. Two characteristics of members of this family are the presence of a disulfide bond in the N-terminal domain of the protein and an amidated C-terminal (34–36). In order to determine the state of oxidation of the disulfide bond between cysteine residues 2 and 7 of rhIAPP, we have carried out DTNB (5,5′-dithiobis-(2-nitrobenzoic) acid) titrations of thiol reactive groups in the recombinant protein according to previously described procedures (37, 38). The results showed the absence of reactive thiols in rhIAPP (data not shown), indicating that the disulfide bond between Cys^2 and Cys^7 is correctly formed in rhIAPP. This result is in agreement with previous studies that showed that oxidation of the cysteine residues of IAPP and formation of the disulfide bond take place rapidly and spontaneously by incubation in dilute solutions at mildly alkaline pH (39).

As shown in Fig. 4, rhIAPP displays a slightly higher electrophoretic mobility when compared with the synthetic polypeptide, which is amidated in its C-terminal. A 1-Da difference in molecular mass was observed (Fig. 5); however, the difference in electrophoretic mobility is more likely to be due to the charge difference between both products (respectively, car-

boxyl *versus* amide groups in the C-terminal), which could lead to different mobilities in the urea-based Tricine electrophoretic system.

The presence of a C-terminal negative charge is likely to affect the charge distribution of the protein and could affect its biological and cytotoxic properties. Although C-terminally amidated IAPP has been shown to be more potent than non-amidated IAPP in terms of its biological actions (*i.e.* in carbohydrate metabolism, Ref. 40), amidation is not essential for the biological functions of this peptide. We also note that the cytotoxicity of rhIAPP was very similar to that of amidated, synthetic IAPP (Fig. 7). Furthermore, the kinetics of aggregation of rhIAPP was in excellent agreement with the results obtained using synthetic, amidated IAPP.[2] Finally, the morphology of the amyloid fibrils formed by recombinant IAPP (Fig. 6*C*) is very similar, if not identical, to the morphology of amyloid fibrils formed by synthetic, amidated IAPP under the same conditions (not shown). These observations lead us to believe that, although non-amidated, rhIAPP recapitulates several important properties of mature human IAPP.

Taken together, the results described here show that we have successfully generated recombinant human IAPP that exhibits *in vitro* amyloidogenic and cytotoxic properties which are very similar to those of synthetic IAPP. Given the strong correlation between IAPP amyloid deposits and type II diabetes mellitus, one of the major goals of our study was to provide a simple procedure to obtain recombinant IAPP for studies aimed at investigating its cytotoxic properties in islet cells. The availability of a reliable protocol for the production of recombinant proteins that have the tendency to form amyloids may be generally useful in studies of other amyloidogenic proteins and/or peptides and in developing approaches to prevent disease-associated aggregation.

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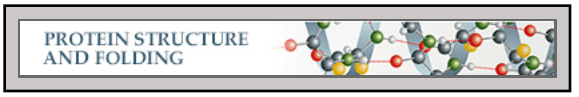
[2] D. H. J. Lopes, unpublished results.

Protein Structure and Folding:



Amyloidogenicity and Cytotoxicity of Recombinant Mature Human Islet Amyloid Polypeptide (rhIAPP)

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